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Doctorate thesis
Role of pericytes in the barrier task
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List of abbreviations

- BBB……………………..…………... blood brain barrier
- BCPs…………………….....bovine cochlear pericytes
- BLB ……………………………...blood–labyrinth barrier
- BM…………..………………..……...basement membrane
- BRB …………………………………blood-retinal barrier
- CNS ……………..……………...central nervous systems
- DR …………….…………………diabetic retinopathy
- DCFDA……………..…2',7'-dichlorofluorescin diacetate
- DME ………………………………diabetic macular edema
- ECs…………………………………….. endothelial cells
- EP ……………..……………….…..endocochlear potential
- FGF-2……………..……....………fibroblast growth factor-2
- hASCs ………..…………....human adipose stem cells
- hRECs……………..……human retinal endothelial cells
- hRPCs ………….…..……………human retinal pericytes
- JNK ………………..…….…………Jun Nterminal kinase
- LDH …………………………..lactate dehydrogenase
- LPS……………..……………………. lipopolysaccharide
- MAPK …..……….……mitogen-activated protein kinase
- MMP…………….…………………..….metalloproteinases
- MSCs…………………….…..Mesenchymal Stem Cells
- NG$_2$…………….…………………….…..neural/glial antigen 2
- PCs …………….….……………………….pericyte cells
- PDGF-B……….……….. platelet-derived growth factorB
- PGS………..….……………pericyte growth supplement
- PIGF ………….………..…………placental growth factor
- ROS…………………….…..reactive oxidative species
- TEER…………..trans-endothelial electric resistance
- TJs ……………….……..………………..tight junctions
- TNF…………….…..……………….. tumor necrosis factor
- VEGF ……………vascular endothelial growth factor
- α-SMA……………..…………. alpha smooth muscle actin

Abstract

The function of endothelial-blood/tissue barrier is critical for maintaining and controlling tissue homeostasis. The blood-retinal barrier (BRB) is a blood-ocular barrier system, made up of an inner and an outer barrier. BRB is located in the retinal microvasculature and include the microvascular endothelium. The role of the BRB is to control molecule trafficking: ion, protein and water flux, from the blood to the retina. Another barrier is the cochlear blood-labyrinth-barrier, located in the *stria vascularis* (intrastrial fluid-blood barrier) that controls the permeability and maintains a stable homeostatic system in the inner ear, between the systemic circulation and the fluids inside the *stria vascularis,* by regulatory mechanisms that are, however, largely unknown. The blood-labyrinth barrier in the *stria vascularis* is anatomically different from the blood barriers in the retina. In contrast to the high degree of vascularization and loose association with surrounding non vascular tissue present in other barriers, the blood-labyrinth barrier is a multi-layer barrier with a network of dense capillary system of marginal epithelial cells with surrounding pericyte cells (PCs) and a basement membrane of mesodermal basal cells, interconnected by tight junctions. In both barriers the PCs have a crucial role: their functions are linked to regulating cerebral blood flow, barrier permeability, cerebral vascular formation maintenance and regulating. It was seen that PCs are involved in vascular development, integrity, angiogenesis and tissue fibrogenesis. The loss of PCs contributes to the pathogenesis of many disorders such as an early feature of diabetic retinopathy, characterised by retinal microvascular dysfunction and degeneration in the BRB. In the physiology of the cochlea, PCs play a role in numerous cochlear pathologies, including, but not limited to, sudden sensorineural hearing loss, acoustic trauma, and inflammation of the cochlea. Understanding the role of PCs in pathophysiology, particularly how it relates to microvascular degeneration and pathological neovascularisation, is critical in order for the development of novel therapies to complement, or improve upon, current treatment options. This research evaluates the potential of human adipose stem cells (hASCs) in differentiating into the phenotype of PCs and examines the effects of hASC like-pericytes on human retinal endothelial cells (hRECs) for barrier formation by measuring trans-endothelial electric resistance and by immuno-analysis. Therefore, adipose stem cells-based therapeutic approaches may be usefully exploited to restore retinal microvasculature integrity. A second study was carried out to obtain and cultivate

primary bovine cochlear pericytes (BCPs) and to test the effects of some ototoxic drugs on these BCPs. The use of BCPs for the analysis of ototoxic drugs can be a model to investigate pharmacological mechanisms and protection systems against different insults.

1 General Introduction

A barrier is "a fence or other obstacle that prevents movement or access"; the blood-neural barriers represent dynamic fences on the interface between blood and many districts of central nervous systems (CNS). The specialized blood-neural barriers, as well as the cerebral microvascular barrier, are recognized in the retina, inner ear, spinal cord, and cerebrospinal fluid. These barriers protect the brain against the infiltration of harmful substances and regulate the permeation of beneficial endogenous substances between the blood and the extracellular fluid of the brain. They can also present a major obstacle in the passage of drugs, peptides and endogenous compounds, protecting neural tissues. Microvascular endothelial cells (ECs) in the brain closely interact with other accessory cells such as astrocytes, pericyte cells (PCs), perivascular microglia and neurons to form a functional 'neurovascular unit'. Homeostasis of the brain is enhanced and maintained by communication between ECs and other surrounding cells. Cell-to-cell communication in the blood-neural barriers is important to development and to maintain barrier integrity and avoid alteration of the blood-neural barriers and cerebrovascular disorders with disruption of the neurovascular unit (Choi et al., 2008). However, the physiology of many blood–neural barriers is largely unknown and the mechanisms of control are poorly understood. To study the physiology of these biological dynamic separations, researchers have also developed *in vitro* cell-culture models that can be employed to investigate transport and metabolism at the barriers and to study cell–cell interactions and regulation of blood barrier permeability (Dupor et al., 1998; Lai and Kuo, 2005). The blood retina barrier represents one of the neurovascular units. It has active functions in brain-retinal microvascular homeostasis and it is involved in the structural integrity of the retina. The blood-retinal barrier (BRB) in the retina can be divided into two distinct regions: the inner BRB and the outer BRB. The inner BRB, formed by the specialized microvessels of the retina and their surrounding PCs and astrocyte end-feet, plays essential roles in protecting neural tissues from toxic materials and in maintaining the neural functions of retina. Tissue integrity and barrier regulation function and cell–cell communication are insured by interendothelial complex junctional structures,

namely adherens junctions, tight junctions (TJs) and gap junctions. The most highly enriched TJ protein is claudin-5, which is critical in mediating the diffusion of molecules in the inner BRB. TJ disruption is observed in numerous retinal degeneration pathologies (Choi et al., 2008; Kim et al., 2016). The retina is known to have the highest density of PCs in the body. PCs play a substantial role in the development of barrier TJs and paracellular permeability by secreting transforming growth factor-β and angiopoietin-1. Similarly, the loss of PCs in the retina has been recently linked to the breakdown of the BRB and to the infiltration of inflammatory cells. Furthermore, it has been seen that the loss of PCs, apparently irreversible, is one of the earliest hallmarks of diabetic retinopathy (DR), a microvascular complication of diabetes in which retinal blood vessels become weakened and break. Pericyte loss causes exudative leakage of fluid into the *macula*, resulting in macular edema or the formation of new vessels, resulting in proliferative DR (Stitt et al., 2013). There is a close interaction between PCs and ECs and pericyte coverage on endothelial cell is higher than in any other tissue (Ejaz et al., 2008). The interactions between PCs and ECs play an important role in blood vessel formation, stability and permeability. PCs promote endothelial sprouting, which results in the loss of side branches and the enlargement of vessels when pericyte function is impaired or lost. PCs are involved in angiogenesis by the secretion of vascular endothelial growth factor (VEGF) and interleukin-6 facilitates maturation of ECs and microvessel spouting, which play an important role in the early stage of angiogenesis. Today, the therapies for DR include laser photocoagulation vitreoretinal surgery, intravitreal injection of VEGF neutralizing agents or corticosteroids, but these therapies can only tone down the progression of the disease. In recent years, stem cell-based approaches have been used to limit pericyte loss in DR (Kim et al., 2016). In particular, human adipose stem cells (hASCs) have been widely investigated in the last few decades for their multipotent differentiation ability and used as a tool for potential therapeutic applications in a variety of diseases (Lo Furno et al., 2016). In the present investigation, the differentiation of hASCs toward a pericyte phenotype was tested by *in vitro* cultures. As a matter of fact, PCs share several mesenchymal stem cell (MSCs) features (Birbrair et al., 2017). In this work, the expression of alpha smooth muscle actin (α-SMA) and (neural/glial antigen 2) (NG_2) was evaluated by immunocytochemistry as indicative of a pericyte-like differentiation of ASCs. In further steps, co-cultures of human retinal endothelial cells (hRECs) with variously pretreated hASCs or human retinal pericytes (hRPCs) were also prepared to evaluate their possible interactions. Previous co-culture studies showed that PCs can increase levels of junction proteins in endothelial cell cultures and decrease vascular permeability (Ting et al.,

2019). Finally, to evaluate the possibility to develop *in vitro* a sound BRB, trans-endothelial electric resistance (TEER) was measured in the co-cultures of hRECs and pericyte-like ASCs. PCs also play a role in other neurovascular units, such as *stria vascularis*; even in these barriers, PCs are linked to regulating cerebral blood flow, barrier permeability, cerebral vascular formation maintenance and neuroinflammation. Many methods of isolation and culture of barrier cells from different tissues are currently available but they are limited for the blood-labyrinth barrier (BLB), due to the difficulty of isolating cells from the vestibular system. The *stria vascularis* is also known as the blood-labyrinth barrier and is structurally and functionally similar to the blood-brain barrier and plays an important role in acoustic trauma and drug-mediated hearing loss. The intrastrial fluid–blood barrier represents a homeostatic system of the inner ear, which tightly regulates the trafficking of molecules from and to the bloodstream and maintains the endolymph high potassium (K^+) levels required for endocochlear potential (EP) (Hibino and al., 2010; Quraishi and Raphael, 2008; Salt et al., 1987). Dysfunction of the intrastrial fluid-blood barrier is considered to be one of the etiologies in a number of hearing disorders, including autoimmune inner ear disease, noise-induced hearing loss, age-related hearing loss, and genetically linked hearing diseases. Despite the importance of the intrastrial fluid-blood barrier, the physiology of the barrier is largely unknown as is how strial PCs contribute to the composition and formation of the strial barrier because it has not been specifically studied but it was seen that the accessory cells, such as PCs, play an important role in the intrastrial fluid-blood barrier. In the stria, PCs are involved in vascular development, and integrity, angiogenesis, and tissue fibrogenesis, and provide mechanical strength and physical elasticity to the vessels (Takeuchi et al., 2001). Furthermore, they show a great capacity to affect intrastrial fluid-blood barrier integrity through the regulation of TJs between ECs, becoming essential for intrastrial fluid-blood barrier integrity (Takeuchi et al., 2001; Yang et al., 2011). Recently, it has been shown that PCs are particularly susceptible to proinflammatory stimuli; for example, the reduction of the precapillary diameter, which strongly contributes to blood flow impairment in the *stria vascularis,* is related to PC rearrangement induced by tumor necrosis factor (TNF) (Bertlich et al., 2017); furthermore, following lipopolysaccharide (LPS) treatment, PCs display a prominent round body and peel away from the capillary wall, strongly contributing to barrier breakdown (Zhang et al., 2015). *Stria vascularis* is target of damage induced by a variety of drugs. In this study, we describe a novel growth medium-based method associated with a mild enzymatic digestion to obtain primary PCs from the *stria vascularis* of bovine cochlea explants. Furthermore, we evaluated the sensitivity of

primary BCP to three potentially ototoxic drugs: furosemide, gentamicin and cisplatin, at different concentrations.

2 Blood Barriers

Blood flows through an elaborate vascular network supplies oxygen and nutrients into many districts of CNS; the exposure to blood occurs through barriers. The CNS barriers are not absolute barriers that block all transport across the endothelium. The specific characteristics of the vasculature in these tissues provide the basis of highly selective barrier transport that regulates the flux of ions, water, solutes or cells across the vascular bed. The function of endothelial-blood/tissue barrier is critical for maintaining and controlling tissue homeostasis and for protecting the neuronal tissue environment from potential toxicity. Both vascular and neuronal diseases, such as stroke, Alzheimer's disease, and Huntington's disease, have been linked with disrupted neurovascular interactions and a failed blood-brain barrier (BBB) (Sweeney et al., 2018).

2.1 Blood Retinal Barrier

The BRB restricts permeability between the blood and the retina: neural and glial cells gain metabolic support from a unique vascular structure that provides the necessary nutrients, while minimizing interference with light sensing (**Box 1**). Neuron-specific perturbations are possible in the retina, as a part of the CNS, involving the structural integrity of the BRB, which separates the retina from the choroid by Bruch's membrane. Structurally, the BRB is made up of two distinct areas: the outer and the inner BRB (**Fig. 1**). The outer consisting of a retinal pigment epithelium that regulates transport between the chorio-capillaris and the retina. That inner regulates transport across retinal capillaries. The ECs of the inner retinal capillaries are not fenestrated, whereas those of the choroidal capillaries are. The inner BRB, similar to the BBB, is located in the inner retinal microvasculature and includes the microvascular endothelium, which lines up these vessels. The barrier consists of tightly interconnected capillary ECs covered with PCs and glia. The TJs, located between these cells, contribute to increase the selectivity of the diffusion of molecules from the blood to the retina and the barrier is essential to maintain retinal homeostasis. Breakdown of the inner BRB has been demonstrated to contribute to the pathophysiology of a number of retinal diseases including DR (Yang et al., 2011). There is a connection between PCs and ECs. PCs play a substantial role in the development of barrier TJs and paracellular permeability by secreting transforming growth factor-β and angiopoietin-1. Similarly, the loss of PCs in the retina has been recently linked to the breakdown of the BRB and to the infiltration of

inflammatory cells. The BRB is particularly rich in TJs and endothelial TJs, which have been mostly studied in these locations (**Box 2**). It is currently admitted that the BRB TJ phenotype is induced by two perivascular cell types: astrocytes through their cellular processes and the closely associated PCs. Both cells produce factors, including angiopoietin-1, TGFβ and probably other uncharacterized molecules, which are required to induce the barrier phenotype (Close et al., 2004; Dohgu et al., 2005; Dohgu et al., 2004). Occludin is associated with increased TJ barrier function. In the endothelium, this molecule is specifically expressed in the BRB (Hirase et al.,1997) and its expression is increased with pericyte-derived angiopoietin-1 (Hori et al., 2004). Occludin downregulation has been observed in various disease states associated with BRB disruption, including stroke, diabetes as well as hypoxia/aglycemia (Wallez and Huber, 2008).
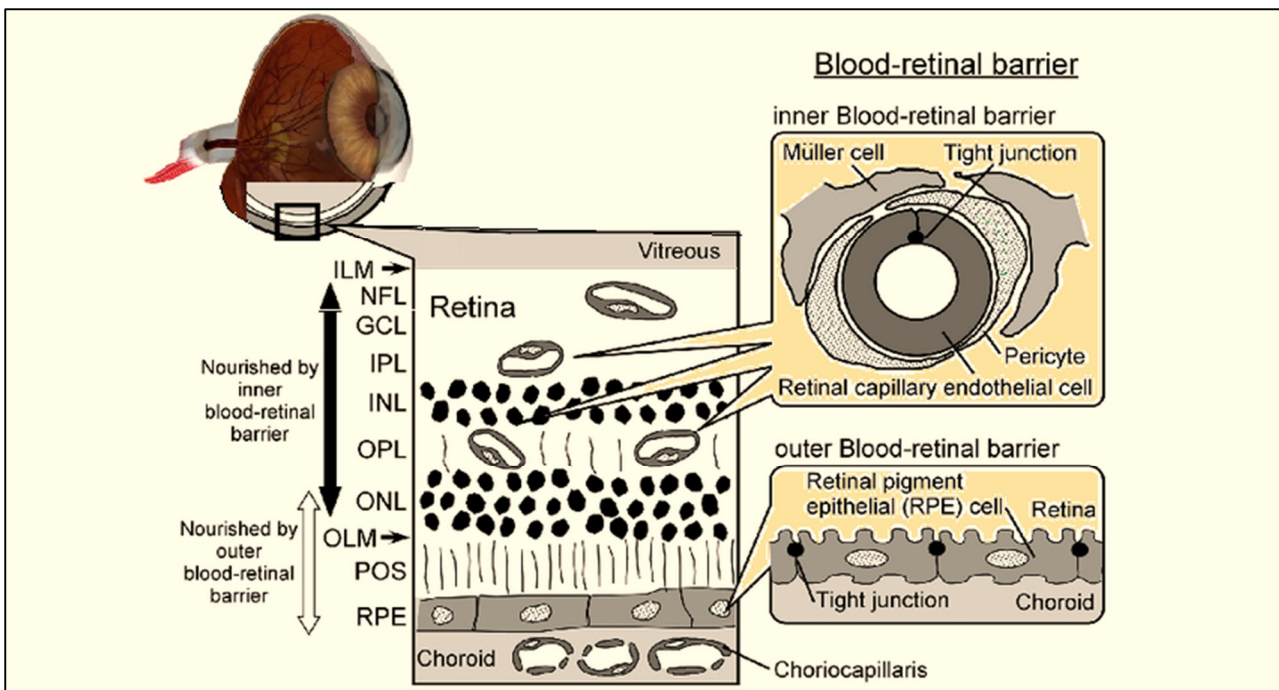

Fig. 1 Schematic Diagram of the inner and outer BRB

Box 1 Functional organization of the retina network

The retina is a thin, delicate, transparent sheet of tissue derived from the neuroectoderm. It is partitioned from the systemic circulation by the blood-retinal and blood-aqueous barriers and receives its nutritional supply from the retinal and choroidal circulations. It is a transparent layer of neural tissue between the retinal pigmented epithelium and the vitreous body. The retina contains a nonsensory layer (retinal pigment epithelium) and neurosensory retinal layers (neuroretina) (**Fig. 2**).

Nonsensory Layer of the Retina: the photoreceptors reside in the retinal pigment epithelium (RPE), a monolayer of cuboidal cells characterized by a high concentration of melanosome, cells with a large amount of melanin pigment in the cytoplasm, from which the cells derive their pigmented color. This allows it to absorb light, which then reaches the retina. RPE is a multifunctional layer. The apical cell of the RPE is closely linked to the photoreceptors, which form a true functional unit. On the other side, the RPE forms a complex with Bruch's membrane of the choroid; therefore, its attachment to the choroid is strong. Despite its close association with the choroid, the RPE is considered a part of the retina because it is from the same embryologic germ cell layer-neural ectoderm. The RPE cells help to nourish the overlying neurosensory retina, facilitating the diffusion of nutrients from the choroid and the removal of waste or worn-out photoreceptor segments. The apical portion of an RPE cell consists of microvilli that extend into the layer of photoreceptors; however, no intercellular junctions connect the RPE and photoreceptor cells. A potential space separates the epithelial cell and the photoreceptor.

Neurosensory Retinal Layers or neuroretina, consists of outer and inner segments of photoreceptors (rods and cones), external limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and internal limiting membrane (Gupta et al., 2015). There are five types of neurons in the retina: photoreceptors, bipolar cells, ganglion cells, horizontal cells and amacrine cells. The cell bodies and processes of these neurons are stacked in the five alternating layers. Light must traverse these layers before initiating signal transduction in the rods and cones. Normal vision depends on intact cell-cell communication among the neuronal, glial, microglial, vascular and pigmented epithelial cells. The retina contains the first three cells (photoreceptor, bipolar and ganglion) in the visual pathway. Absorption of light by the photopigment in the outer segment of the photoreceptors initiates a cascade of events that changes the membrane potential of the receptor, and therefore the amount of neurotransmitter released by the photoreceptor synapses onto the cells they contact. This is the site of transformation of light energy into a neural signal by capturing photons, converting the photochemical energy into electrical energy through the process of phototransduction, transferring this signal to bipolar cells. The synapse with ganglion cells transmits and integrates the resulting action potentials from the eye to the occipital lobe of the brain travelling along axons in the optic nerve. In the brain the informations are deciphered and interpreted into recognizable images. Other retinal cells, horizontal cells, amacrine cells and interplexiform neurons, modify and integrate the signal before it leaves the eyes (Remington, 2012; Purves et al., 2001).

The **photoreceptor layer** is formed by rods and cones composed of the outer and inner segments of the rod and cone cells. The outer segment contains stacks of membrane discs, which enclose visual pigment molecules and are constantly renewed. The **external limiting membrane**, separating the inner and outer segments from the photoreceptor nuclei, is not a true membrane but a series of dashes formed by the terminal bar attachments of the cell bodies of rods, cones, and

Müller cells. The **outer nuclear layer** contains the nuclei of the photoreceptor cells (both rod and cone cells). The **outer plexiform layer** is formed by the axons of the photoreceptor cells and their synapses with bipolar cells. The **inner nuclear layer** contains the nuclei of bipolar cells. Bipolar cells are the first-neuron cell to process the electrical stimulus coming from the photoreceptors before transmitting it to the ganglion cells.

They connect the photoreceptor cells ultimately with the dendrites of the ganglion cells in the inner plexiform layer. In addition, it includes horizontal interneuron cells, mainly GABAnergic neurons, which adjust vision in extreme environmental light conditions (Gupta et al., 2015; Purves et al., 2001).**The inner plexiform layer** is composed of synapses between the bipolar, ganglion, and amacrine cells (responsible for adjusting the retinal image). Amacrine cells receive excitatory glutamatergic input from bipolar cells and primarily inhibitory input from other amacrine cells mediated by GABAC receptors. These cells can synapse back on to bipolar cells, other amacrine cells and ganglion cells. Müller cells act as specialized glial cells to form retinal scaffolding, support the inner segments of the photoreceptors and create the acellular fibrous internal limiting membrane. The **ganglion cell layer** are the second-neuron cell in visual via and comprises cells that transmit impulses from the photoreceptors, through their long axons, to the thalamus. The **nerve fiber layer** is formed by the ganglion cell axons (Romero-Aroca et al., 2016).
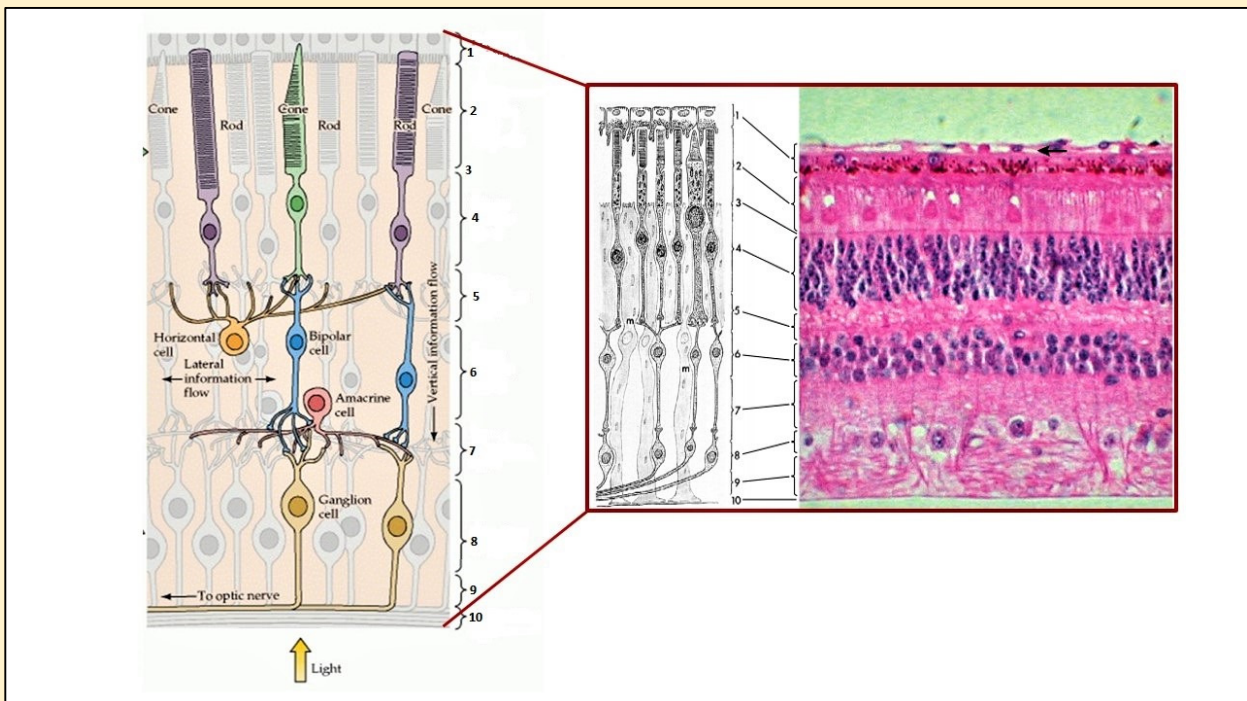

Fig. 2 Structure of the retina and section of the retina showing overall arrangement of retinal layers:

1, retinal pigment epithelial layer;

2, photoreceptor layer;

3, external limiting membrane;

4, outer nuclear layer;

5, outer plexiform layer;

6, inner nuclear layer;

7, inner plexiform layer;

8, ganglion cell layer;

9, nerve fiber layer;

10, internal limiting membrane

Box 2 Protein components of the intercellular junctions

Interendothelial junctions contain complex junctional structures, namely adherens junctions (AJs), TJs and gap junctions (GJs), playing pivotal roles in tissue integrity, barrier function and cell–cell communication, tight and adherens junctions play a central role in barrier regulation. In ultrathin sections of epithelial cells, TJs appear as contact points of close apposition, or "kissing points", where the two lipid bilayers are indistinguishable; these are located specifically at the most apical side of the polarized lateral membrane (Farquhar and Palade, 1963). The BRB is particularly rich in TJs and endothelial TJs have been mostly studied in these locations. It is currently admitted that the BRB TJ phenotype is induced by 2 perivascular cell types: astrocytes through their cellular processes and closely associated pericytes. Both cells produce factors, including angiopoietin-1, TGFβ and probably other uncharacterized molecules, which are required to induce the barrier phenotype (Ohtsuki et al., 2008; Dohgu et al., 2005; Dohgu et al., 2004). The presence of TJs between the ECs of the iBRB helps to mediate the very low passive permeability of the tissue, permitting entry of nutrients into the retina but excluding harmful toxic material and inflammatory cells. In numerous retinal degeneration pathologies, TJ disruption is observed, and a more refined understanding of this disruption could be used for therapeutic benefit. At the molecular level, TJs consist of many proteins which can be categorized: transmembrane proteins, including the tetraspanin families of claudin and junctional adhesion molecule (JAM) family cytoplasmic-scaffold proteins, such as zonula occludens (ZO) (Bazzoni et al., 2000). The claudin family are tetraspan transmembrane proteins that contribute a crucial role in paracellular transport by forming ion selective barriers and pores. In addition, the carboxy-terminus of claudins interacts with the first of three PDZ domains on ZO-1 (Itoh et al., 1999). ZO-1 allows the connection between transmembrane proteins and the actin cytoskeleton, and controls the localization of claudins at cell contacts. Claudin-5 is the most abundant, 500-fold more abundant, than the other claudin subtypes (Ohtsuki et al., 2008). It localizes at the TJs of retinal vessels, together with claudin -1 and -2. These proteins may be part of organized junctional structures, such as claudins and occludin in TJs, VE–cadherin in AJs, or connexins in GJs, while others are independent. JAMs are associated with TJs through intracellular components without being directly involved in TJ strand formation. Another

transmembrane component of TJ strands is occludin. Although not necessary for TJ strand formation, occludin is associated with increased TJ barrier function. In the endothelium, this molecule is specifically expressed in the BRB (Hirase et al., 1997) and its expression is increased with pericyte-derived angiopoietin-1 (Hori et al., 2004). Occludin downregulation has been observed in various disease states associated with BRB disruption, including stroke, diabetes as well as hypoxia/aglycemia (Wallez and Huber, 2008). Decreased occludin contents together with increased paracellular permeability were observed in VEGF-treated retinal endothelial cells. These features suggest that occludin degradation may be one of the mechanisms increasing vascular permeability. Claudins and occludin are linked to numerous intracellular partners, including Zonula occludens (ZO): ZO-1, ZO-2 and -3. They are large (>200kDa) scaffold proteins that connect transmembrane proteins with the cytoskeleton. ZO proteins form a molecular complex by their ability to bind actin, α-catenin, and afadin (AF6) (Itoh et al., 1999) and play an important role in TJ organization. The ZO-1α– isoform is only observed in endothelial cells, and it is characterized by more dynamic junctions (Wallez and Huber, 2008). Adherens junctions (AJ) play a critical role in cell-cell adhesion, cell polarity, contact inhibition, and paracellular transport regulation. The adherens junctions of the iBRB includes (VE)-cadherin of the cadherin superfamily. VE-cadherin is a transmembrane Ca^{2+}-dependent cell adhesion protein with a conserved cytoplasmic tail that binds to β-catenin (Carmeliet et al., 1999). VE-Cadherin has an intimate relation to the VEGFR and is a major control point for regulation of barrier development, paracellular permeability and growth control (Díaz-Coránguez et al., 2017) (**Fig.3**).
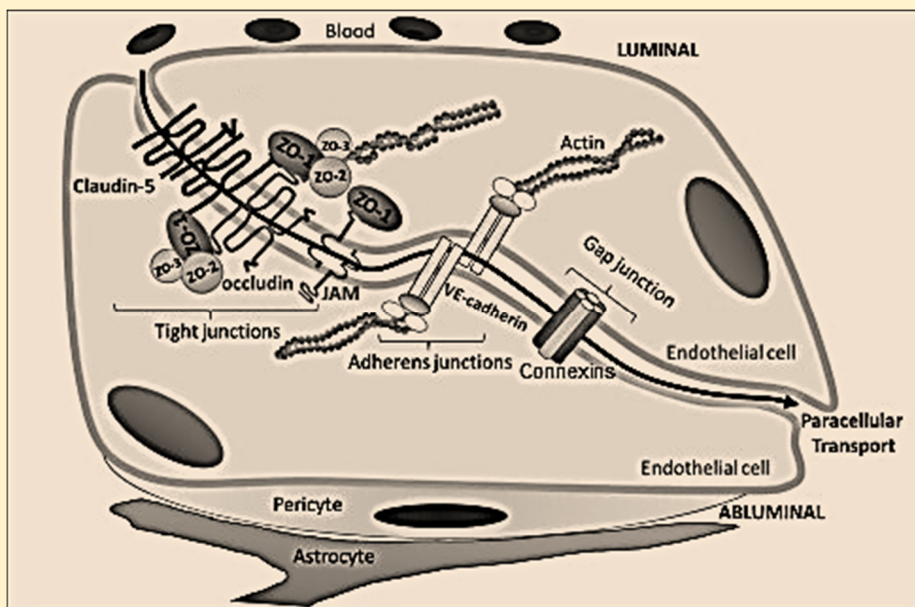

Fig 3 Adhesive proteins within the interendothelial cleft

2.2 Intrastrial Fluid-Blood Barrier

The *stria vascularis* is a cell complex of the inner ear that promotes the process of hearing by generating a positive EP in hair cells of the cochlea and secretes K^+. K^+ is the main charge carrier and the EP is the main driving force for the sensory transduction that leads

to hearing (**Box 3**). This is a highly complex tissue, composed of three types of different cell types located peripherally, some of which line the blood capillary space with extensive cytoplasmic thin protrusions towards the ECs that are centrally located. The *stria vascularis* is also known as the intrastrial fluid-blood barrier and is structurally and functionally similar to the blood-brain barrier, but it is anatomically different from blood barriers in the retina (**Fig 4**) the intrastrial fluid-blood barrier consists of two parts, marginal cells that secrete potassium and basal cells that are coupled via gap junctions to intermediate cells (Marcus et al., 2002). It comprises a network of dense capillaries of ECs characterized by a unique polygonal organization with surrounding PCs and basement membrane (BM) (Takeuchi et al., 2001; Shi, 2016). In contrast to the high degree of vascularization and loose association with surrounding nonvascular tissue present in other barriers, the BLB is a multi-layer of epithelial marginal cells and mesodermal basal cells interconnected by TJs. Dendrite-like projections of intermediate cells, dispersed between the two cell layers, make close contacts with capillaries through GJs. The complexity of the capillary beds contribute to isolation of *stria vascularis* cells from surrounding non-vascular cell. The *stria vascularis* is involved to acoustic trauma and drug-mediated hearing loss. The intrastrial fluid-blood barrier maintains a stable homeostatic system of the inner ear by regulatory mechanisms, including control over ion, fluid, and nutrient active and passive transport. Normal function of the stria is crucial to maintain the ionic gradients and EP required for sensory hair cell transduction (Yang et al., 2011; Frey and Antonetti, 2011).
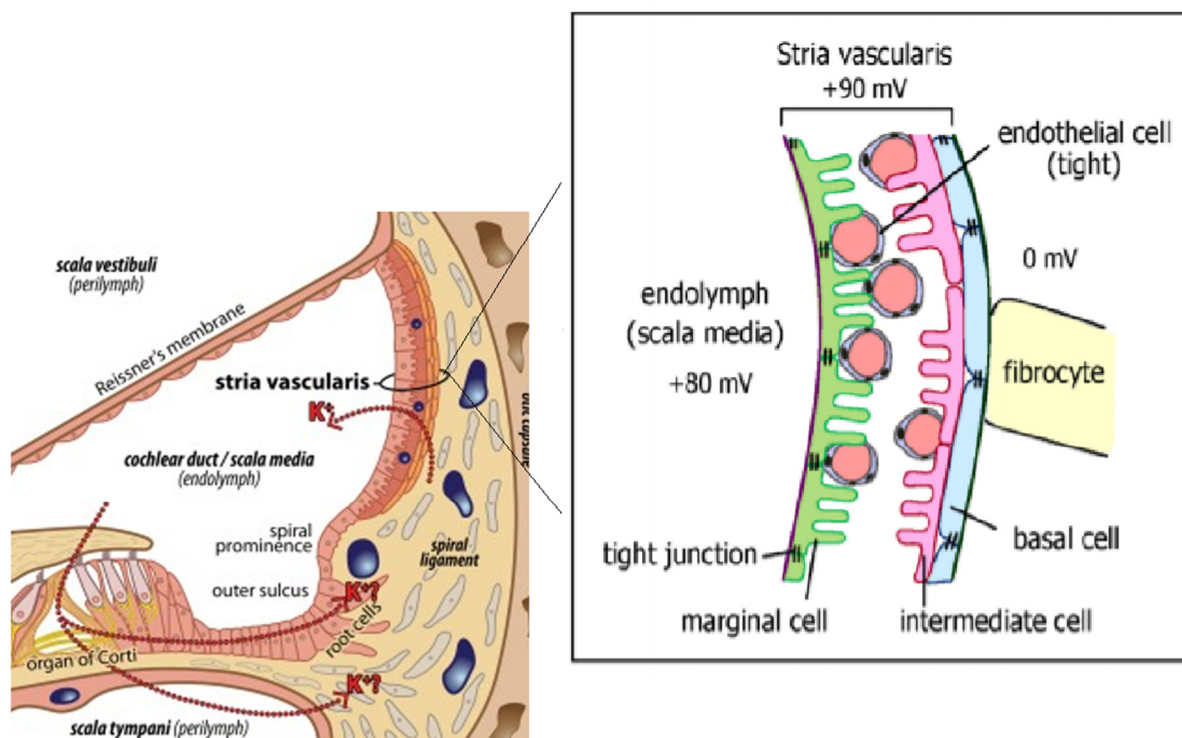
Fig 4 Intrastrial fluid-blood barrier

Box 3 *Stria vascularis*
Cochlear sensory hair cells mediate the senses of hearing, balance, linear acceleration and angular acceleration (McPherson, 2018). They are responsible for converting sound into electrical signals via the cochlear nerve (Hudspeth, 2005). In the mature mammalian cochlea, the main terminal artery is the spiral modiolar artery, a secondary branch of the anterior inferior cerebellar artery. The two major microvessel networks in the cochlea are the vessels of the *stria vascularis* and the vessels of the spiral ligament. The positive Endocochlear Potential (EP) in the endolymph of the cochlear duct in hair cells is generated by a unique composition of high potassium ions (K^+) and low sodium. Specialized cells in the lateral wall of the cochlear duct (*scala media*), at the level of the *stria vascularis*, secrete these ions into the endolymph. The *stria vascularis* synthesizes and secretes endolymph and maintains ionic composition (Patuzzi, 2011), while the role of the vessels of the spiral ligament is less well understood (Shi et al., 2008). The EP of about +80 mV in conjunction with the high K^+ concentration ($[K^+]$) of endolymph of about 150 mM drives the sensory transduction in the hair cells that leads to hearing (Canis and Bertlich, 2019). The molecular mechanisms of K^+ secretion in strial marginal cells have been unambiguously determined, whereas the molecular mechanisms that lead to the generation of the EP remain unknown. Some evidence led to the hypothesis that KCNJ10 (Kir4.1) potassium channel in intermediate cells generates the EP. The *stria vascularis* is a highly vascularized, multi-layered epithelium adjacent to the spiral ligament in the lateral wall of the cochlea and consists of two epithelial barriers, marginal cells and basal cells. The melanocytes, intermediate cells, are the third type of cells. Marginal cells with an epithelial origin, border the lumen of the cochlear duct, they transport potassium into scala media, however endocochlear potential cannot be generated by the marginal cells alone;

their function is involving passive potassium movement across the apical membranes of the basal cells (Steel et al., 1989; Salt et al., 1987). Intermediate melanocyte-like cells, originating from the neural crest, are disseminated between the marginal and basal cell layers; basal cells, from mesodermal or neural crest cells, form a continuous layer. In the stria, the three type of cells are interdigitated with each other (Steel et al., 1989). Anatomical (upper half) and compartmental (lower half) model of the adult *stria vascularis* showing the three cellular layers and depicting the location of potassium regulating channels. The basal cells and intermediate cells are closely linked via a high density of GJs. The intrastrial space is at +80 mV for the high density of KCNJ10 channels in the intermediate cell membrane and the large K^+ concentration ([K^+]) difference between intermediate cell cytosol and the intrastrial space. EP is generated by this voltage and keeps the intrastrial
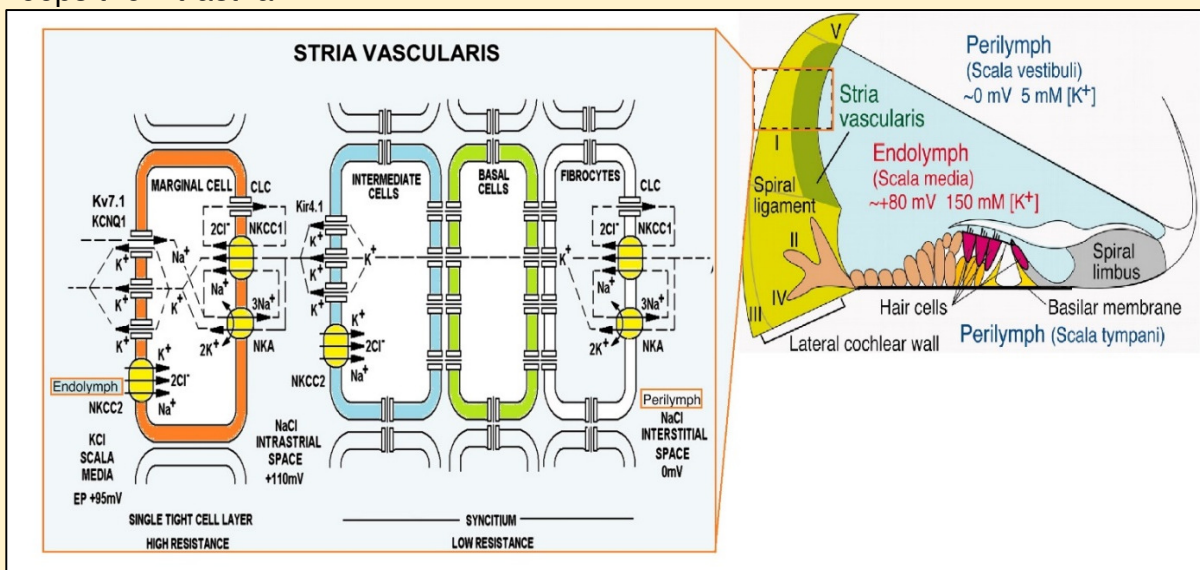

Fig. 5 Cell model of *stria vascularis*

3 Pericytes

While the functional roles of PCs are currently not fully understood, it is widely accepted that they help to stabilize the vessel wall and prevent vascular leakage. The loss or detachment of PCs has been implicated in diseases, such as DR. In this disease, PCs are the primarily affected vascular cells, leading to secondary changes of the endothelium and to dysregulated angiogenesis. PCs are perivascular cells with multifunctional activities. They are usually present on the outside surface of ECs of capillaries. The association with ECs forms a functional unit (**Fig. 6**). PCs extend long cytoplasmic processes over the surface of the ECs. There is a BM, between PCs and the ECs, to which PCs make their contribution. Where the BM is absent, at points of contact, there are different types of endothelial/pericyte cell junctions: the "peg-socket": pericyte cytoplasmic projections (pegs) are inserted into EC invaginations (sockets), that is gap junctions, TJs and adherent junctions: microfilaments

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attached to the pericyte plasma membrane in the contiguous EC cytoplasm (Harrell et al., 2018). No intercellular junctions have been identified between PCs. PCs appear, therefore, to be arranged to facilitate and integrate cell communication. It has also been suggested that PCs may reside around the endothelium of large vessels. A single pericyte often communicates with several ECs through these specialized contacts. They may integrate and coordinate neighboring endothelial cell responses. The interactions between PCs and ECs play an important role in blood vessel formation, stability and permeability. Pericytes have both structural and functional heterogeneity and their location is not random, but functionally determined. Interaction between pericytes and ECs is important for the maturation, remodelling and maintenance of the vascular system via the secretion of growth factors or modulation of the extracellular matrix. PCs on the microvessels are the equivalent of larger vessel smooth muscle cells and the distribution of α-smooth muscle actin, as well as the expression of endothelin receptor in PCs, imply a contractile function of PCs with blood flow regulatory capabilities. An important role for PCs in pathology, and neuropathology in particular, has been indicated in a lot of diseases and in DR. PCs share multiple features with MSCs. Most PCs show a lot of cytoplasmic, lysosome-like granules of variable size and appearance. Pericyte granularity may be associated with extent of cytoplasmic lysosomes, and human brain PCs appear to be exclusively granular with a high content of acid phosphatase (Allt et al., 2001; Dore-Duffy and Cleary, 2011; Chen et al., 2015). There is no single molecular marker for PCs, in part due to the varied lineage and pluripotent capability (Trost et al. 2016). Pericyte identification requires a combination of perivascular location, morphology, and at least two pericyte molecular markers (Armulik et al., 2011).
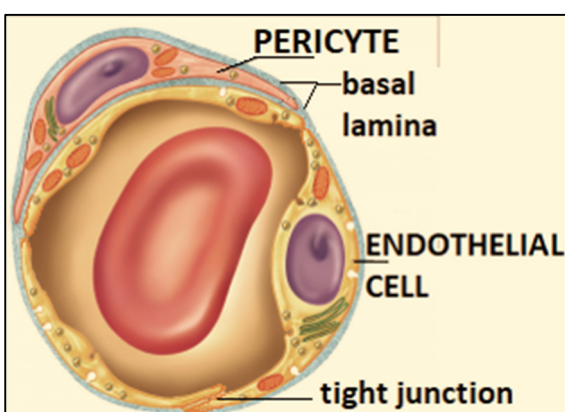

Fig 6 Pericyte and endothelial cell

3.1 Roles of pericytes in the blood-retinal barrier

The early stage of DR is characterised by BRB breakdown, which leads to retinal oedema (the major cause of visual loss in type 2 diabetes), haemorrhage, exudates and capillary microaneurysms. The proliferative phase is the later stage of DR. It is characterised by neovascularization predisposing to vitreous haemorrhage, to fibroplasia with corresponding retinal and vitreous fibrosis, leading to eventual total blindness from tractional retinal detachment. Pericyte degeneration is observed in earliest phases. There is a close interaction between PCs and ECs; the membrane between these cells is thin. In the retina, pericyte coverage on ECs (pericyte/endothelial cell ratio 1:1) is higher than in any other tissue (Ejaz et al., 2008). Many researchers suggest pericyte dropout or loss are possibly causes for first abnormalities observed clinically in DR in the vascular architecture. Their removal from the vascular wall may lead to peripheral endothelial sprouting/pruning and formation of aberrant capillaries, which are early abnormalities in DR (Trost et al., 2016). Pericytes are important regulators in the retina, they have both anti-apoptotic and anti-proliferative effects on ECs and control the expression of several genes (FOXO1, Ang2, and VEGFR2) to protect retinal vessels against injury and stress (Park et al., 2017).

PCs promote endothelial sprouting, which results in the loss of side branches and the enlargement of vessels when pericyte function is impaired or lost. The recruitment of PCs in the developing vasculature is mediated by the release of platelet-derived growth factor B (PDGF-B) by ECs, which activates the corresponding receptor, the tyrosine kinase PDGFRβ, on PCs. PCs are involved in angiogenesis by the secretion of VEGF and interleukin-6 facilitate maturation of ECs and microvessel spouting, which play an important role in the early stage of angiogenesis. VEGF is the key regulator of vasculogenesis, angiogenesis, lymphangiogenesis and vascular permeability in vertebrates. It is recognized as a mediator of blood-retina barrier breakdown, which leads to fluid leakage; VEGF levels, in fact, increase in diabetic human eyes with blood-retina barrier breakdown and neovascularization. While VEGF stimulates directly endothelial cell proliferation and migration, its role in pericyte biology is less clear. Conversely, inhibition of VEGF signaling preserves pericyte survival as well as the integrity of the blood-retina barrier.

PCs regulate VEGF-induced endothelial sprouting through VEGFR1. It has been shown that the expression of vascular endothelial growth factor receptor 1 (VEGFR1) by PCs spatially restricts VEGF signalling. Diabetic macular edema is a major cause of vision loss associated with DR and is characterized by exudation and accumulation of extracellular fluid in the macula secondary to an increase in vascular permeability. Although the pathogenesis of

diabetic macular edema (DME) is multifactorial, including capillary endothelial vascular dysfunction, local inflammatory activity, cellular hypoxia, oxidative stress, breakdown of the endothelial blood–retinal barrier, and retinal neurodegeneration, the overexpression of VEGF has been identified as a key contributor to the development of DME. The proteins VEGF-A, VEGF-B, and the placental growth factor (PIGF) are members of the VEGF family and the most relevant for ocular disease. These proteins, after binding to specific receptors (VEGFR-1 and VEGFR-2), initiate a cascade of events leading to vasculogenesis and angiogenesis (Giurdanella et al., 2015; Eilken et al., 2017).

3.2 Roles of pericytes in the intrastrial fluid-blood barrier

Similar to the structures of the BBB and BRB, the intrastrial fluid-blood barrier includes ECs, PCs, perivascular resident macrophage-like melanocytes (a hybrid cell type with characteristics of both macrophages and melanocytes, structurally and functionally similar to the end-feet of brain astrocytes) and the BM.

The intrastrial fluid-blood barrier is a specialized capillary network that maintains the ionic gradients of the EP required for sensory hair cell transduction, in particular the barrier maintains the endolymph high K^+ levels required for the EP. The accessory cells, such as PCs, play an important role in the intrastrial fluid-blood barrier (**Fig. 7**). The ratio of PCs to ECs on the regions of the capillaries of the *stria vascularis* is between 1:1 and 1:2, which is a relatively high ratio compared to the ratio in most other organs; for example, the ratio of PCs to ECs in the brain is 1:5, in lung 1:10, and in skeletal muscle 1:100. However, the ratio is also high in the retina, where it is 1:1 (Zhang et al., 2015). This high number may be explained by the observation that cochlear capillary PCs may be differentiated into different subgroups in relation of different functions in the physiology of the cochlea: 1) formation of the intrastrial blood-fluid barrier: PCs are involved to homeostasis, monitoring the diffusion of ions, fluid, and nutrients. 2) regulation of cochlear blood flow with their contraction or relaxation, 3) Immune response: PCs actually contribute to the immune response in inflammation of the cochlea (Canis and Bertlich, 2019). Due to these central roles in the physiology of the cochlea, PCs is involved in numerous cochlear pathologies, including, but not limited to, sudden sensorineural hearing loss, acoustic trauma, and inflammation of the cochlea. PCs may be required to control local blood flow and buttress vessel stability in the cochlear vascular system, as has been reported to be the case in the brain and retina (Shi et al., 2008) PCs are distributed in a regional and tissue specific manner, but the distribution

and morphology of PCs in cochlear vascular systems is unknown. The shapes of PCs are different in various locations. The majority of PCs have a polygonal cell body and long, slender processes (Shi et al., 2008). Complex intercellular TJs between ECs show extremely low permeability, limiting passive diffusion of molecules into the cochlea from blood vessels. The strial PCs are notably rich in the structural protein desmin, an intermediate filament protein; by contrast, PCs of the spiral ligament express more contractile proteins such as α-SMA and tropomyosin (Shi, 2009; Franz et al., 2004). Cochlear PC long processes wrapped around capillaries are in close contact with ECs: PCs are embedded in BM where they communicate with ECs through both direct physical contact and paracrine signaling. They generally enhance the integrity of capillary networks in the *stria vascularis*. PCs also contribute to BM formation by directly synthesizing type IV collagen, glycosaminoglycan, fibronectin, nidogen-1, perlecan, and laminin (Fisher, 2009; Shepro and Morel, 1993) and inhibit the activity of destabilizing matrix metalloproteinases (MMP), such as MMP-2 and MMP-9 (Zozulya et al., 2008). However, the contribution of strial PCs to the composition and formation of the strial BM is not yet completely understood. An array of pericyte-related proteins, including α-SMA, desmin, and tropomyosin, are found in the PCs of the *stria vascularis*. The high density of PCs in the microvasculature of the cochlea may be related to functional and metabolic needs in the inner ear. Inflammatory factor-induced hearing disorders are hypothesized to be associated with disrupted vascular integrity in the *stria vascularis* and disturbed endolymph ion homeostasis. The vulnerability to the hypoxia requires an adequate cochlear blood flow to be maintained by cytokines may be one of the causes for the increased permeability of the blood barrier, resulting in homeostatic imbalance and hearing loss.
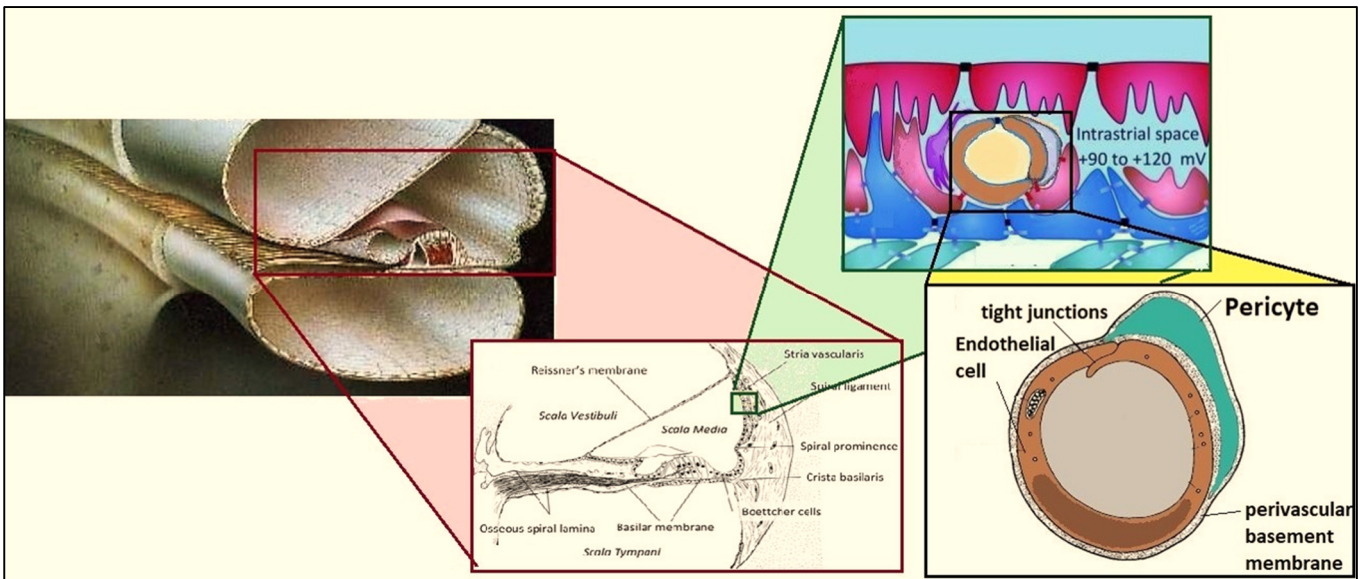
Fig. 7 Pericytes in the Intrastrial fluid-blood barrier

4 Diabetic retinopathy

DR is "structural and functional changes in the retina due to diabetes" (Gardner et al., 2011). DR is a chronic progressive multifactorial disease, a potentially sight-threatening disease of the retinal microvasculature with a complex aetiology. The exact mechanisms by which high blood glucose levels produce diabetes complications are not altogether clear. Nevertheless, it is known that hyperglycaemia has metabolic effects that induce microvascular damage to the retina. Approximately 425 million adults worldwide in 2017 had diabetes mellitus and the number has been projected to grow to 629 million by 2045. Diabetes is one of the main causes of lowering sight or blindness due retinopathy. About 5-10% of worldwide cases of diabetes are classified as type 1 diabetes mellitus. It is due to the autoimmune destruction of the β-cells of the pancreas, which leads to insufficient production of insulin. The type 2 diabetes is linked to peripheral insulin resistance with declining β-cell function and impaired regulation of hepatic glucose production. This type is increased with food availability and caloric consumption associated to a sedentary lifestyle. In 2017, the global prevalence of diabetes in adults aged 20 to 79 years was estimated to be 8.8% and it is estimated that the prevalence of diabetes among people over the age of 16 years will rise by 28.3% between 2010 and 2030, with 54.5% of this increase being attributed to increased obesity.The lifetime risk of developing DR is estimated to be 50% to 60% in those with type 2 and up to 90% in those with type 1 diabetes, thus DR is more common in type 1 than in type 2 diabetes (Tan et al. 2017). Hyperglycemia is a well-known risk factor of both DR and DME in type 1 and

type 2 diabetes (Chen et al., 1995; Tam et al., 2009). A consistent percent-age of patients with DR develop macular edema. DR has historically been considered a retinal vasculopathy, but recently it has seen that it affects the entire neurovascular unit, therefore it is a sensory neuropathy similar to autonomic and peripheral neuropathies that are common features of diabetes and pre-diabetes, including corneal neuropathy. Severe alterations in the retina are seen soon after the development of DR and they due to: microvascular disorder associated with microaneurysms, intraretinal hemorrhages, capillary non-perfusion, intraretinal microvascular abnormalities and neovascularization (**Fig. 8**). DR can lead to vision loss and show the clinical effects in two clinical stages: non proliferative and proliferative DR. Non proliferative DR exhibits damage to retinal vasculature, leaky blood vessels, and associated mobilization of blood components in the retina, while proliferative DR is characterized by a massive retinal neovascularization; new vessels are leaky, fragile, and misdirected and cause poor retinal blood flow. This might progress to vitreous haemorrhage and, in late stages, may cause tractional retinal detachment and neovascular glaucoma (Liu and Arevalo, 2019). The most important finding in DR is the presence of hyperglycemia, which acts on different molecular pathways and damages the BRB, overpowering the cells that make up these structures, such as ECs and PCs.
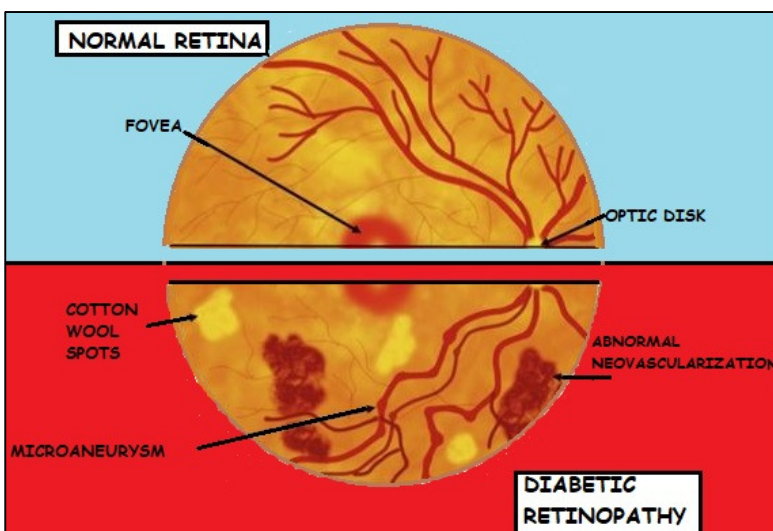

Fig. 8 Structural and functional changes in the retina due to DR

Diabetic patients suffering from DR can show diabetic macular edema that leads to blindness. The VEGF is known to play a role in the development of DME. Angiogenesis and inflammation have been shown to be involved in the pathogenesis of this disease (Romero-Aroca et al., 2016).

4.1 Hyperglycaemia

Hyperglycaemia has been considered in the last decade as the main cause of the onset and progression of DME and DR. Chronic hyperglycaemia and daily fluctuations in blood glucose have been clearly associated with the severity of microvascular complications and it is widely acknowledged that pericyte loss in the first phases of DR is the result of direct hyperglycaemic damage. These events initiate the development of a cascade that culminates in the development and progression of DR.

According to the traditional Brownlee's hypothesis (Brownlee, 2001), reactive oxidative species (ROS) are not only the final effectors, but also the initial ones of the intracellular pathways that are activated in the presence of hyperglycaemia. There are four major biochemical pathways that lead to hyperglycaemic damage of microvascular cells (**Fig. 9**) (**Box 4**).
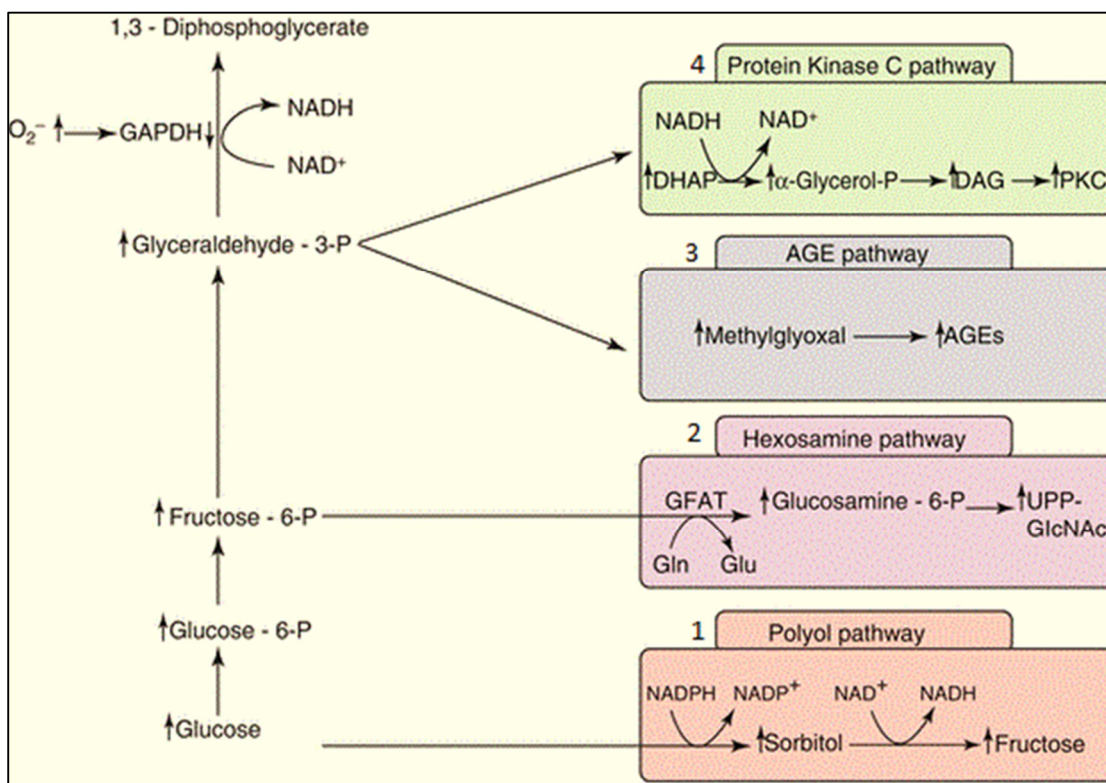

Fig. 9 A possible explanation of hyperglycaemic damage to microvascular cells is the involvement of four major biochemical pathways: 1. increased flux through the polyol pathway; 2. increased flux through the hexosamine pathway; 3. increased formation of advanced glycation end-products (AGE); 4. activation of the protein kinase C (Pfister et al., 2010; Beltramo et al., 2008).

Each of the pathways has been associated with the development of diabetic retinopathy. The interaction between ROS and the previously described pathways defines ROS as playing a key role in DR development, which is difficult to control. The accumulation of free radicals in the form of ROS is linked to histopathological changes such as the thickening of the BM and the loss of ECs and PCs. There is a relationship among these pathways, all of which are linked to the pathogenesis of DR. Oxidative stress contributes to induced inflammatory intermediate production, causes the rupture of the BRB, pericyte demise, and increased vascular permeability, which lead to progression to advanced DR stages and the development of vascular dysfunctions. Increases in glycaemia and ROS overproduction induce phenotypical changes in vascular terms, including retinal ischaemia, high permeability, and retinal neovascularisation induced by the overproduction of VEGF and DME (Behl et al., 2016; Lelkes et al.,1998).

This leads to cellular damage in terms of micro-vascular dysfunction, neuronal apoptosis, glial reactivity and component deposition (Brownlee, 2005; Zhang et al., 2014). All these interconnected aspects are linked to upregulation of angiogenic and inflammatory mediators and to altered growth factor signaling, with recruitment and infiltration of macrophages, monocytes and neutrophils and consequent aberrant inflammatory response. Endothelial dysfunction associated with inflammation determines increased vascular permeability, alteration of blood flow, oxidative stress and angiogenesis and has been related to increased expression of inflammatory adhesion molecules (ICAM-1, VCAM-1 and E-selectin) in the endothelium. Elevated levels of these molecules lead to adhesion and accumulation of leukocytes within retinal vessels. This results in the loss of PCs, formation of acellular capillaries and consequent break-down of the BRB that progresses toward increased retinal vascular permeability, development of DME, and neovascularization (Vujosevic andToma, 2018). Activation of retinal glial cells including astrocytes, Müller cells and microglia is considered another critical feature involved in the initiation and amplification of inflammation in diabetic neuroretinal dysfunction. Activated microglia cells migrate from the inner to the outer retinal layers and produce pro-inflammatory mediators such as TNF-alpha, IL-6, MCP-1 and VEGF, amplifying the inflammatory response that triggers reactive gliosis, a process consisting of hypertrophy, cellular proliferation and an increase in intermediate filament proteins such as glial fibrillary acidic protein (GFAP). The formation of new immature fragile vessels on the surface of the retina in an advanced stage generates vitreous hemorrhages and even tractional retinal detachments, which lead to severe and irreversible loss of vision; retinal hypoxia is a common factor in the development of pathological angiogenesis that is

associated with high levels of VEGF. It is a proangiogenic factor released by the retinal pigment epithelium. VEGF is the most studied factor involved in DR pathogenesis and the main target of available therapeutic strategies. A wide range of systemic and local inflammatory biomarkers are involved and act together in DR; this include vascular adhesion molecules (VCAM-1, ICAM-1, E-selectin, sVAP), pro-inflammatory cytokines (TNF-alpha, IL-1alpha, 1beta, 6, 8, HMGB1), anti-inflammatory cytokines (IL-10), pro-inflammatory/angiogenic chemokines (CP-1, MIF, SDF-1, fractalkine), anti-inflammatory/antiangiogenic chemokines (IP10, MIG), transcription factors (HIF-1, NF-κB), pro-inflammatory/angiogenic growth factors (VEGF, PGF, IGF1, CTGF, stem cell factor), anti-inflammatory/antiangiogenic growth factors (PEDF), anti-inflammatory/proangiogenic growth factors, and innate immune response cells (retinal ECs with toll-like receptors) (Jenkins et al., 2015). In addition, there is some evidence suggesting that pro-inflammatory molecule levels increase with DR progression: in particular, cytokines such as TNF-alpha, VEGF, IL-1beta and IL-6 seem to be associated to DR severity and not only to the presence of DR (Vujosevic andToma, 2018). These events interact to create a pro-inflammatory environment that contributes to the occurrence, maintenance and progression of DR (**Fig. 10**).
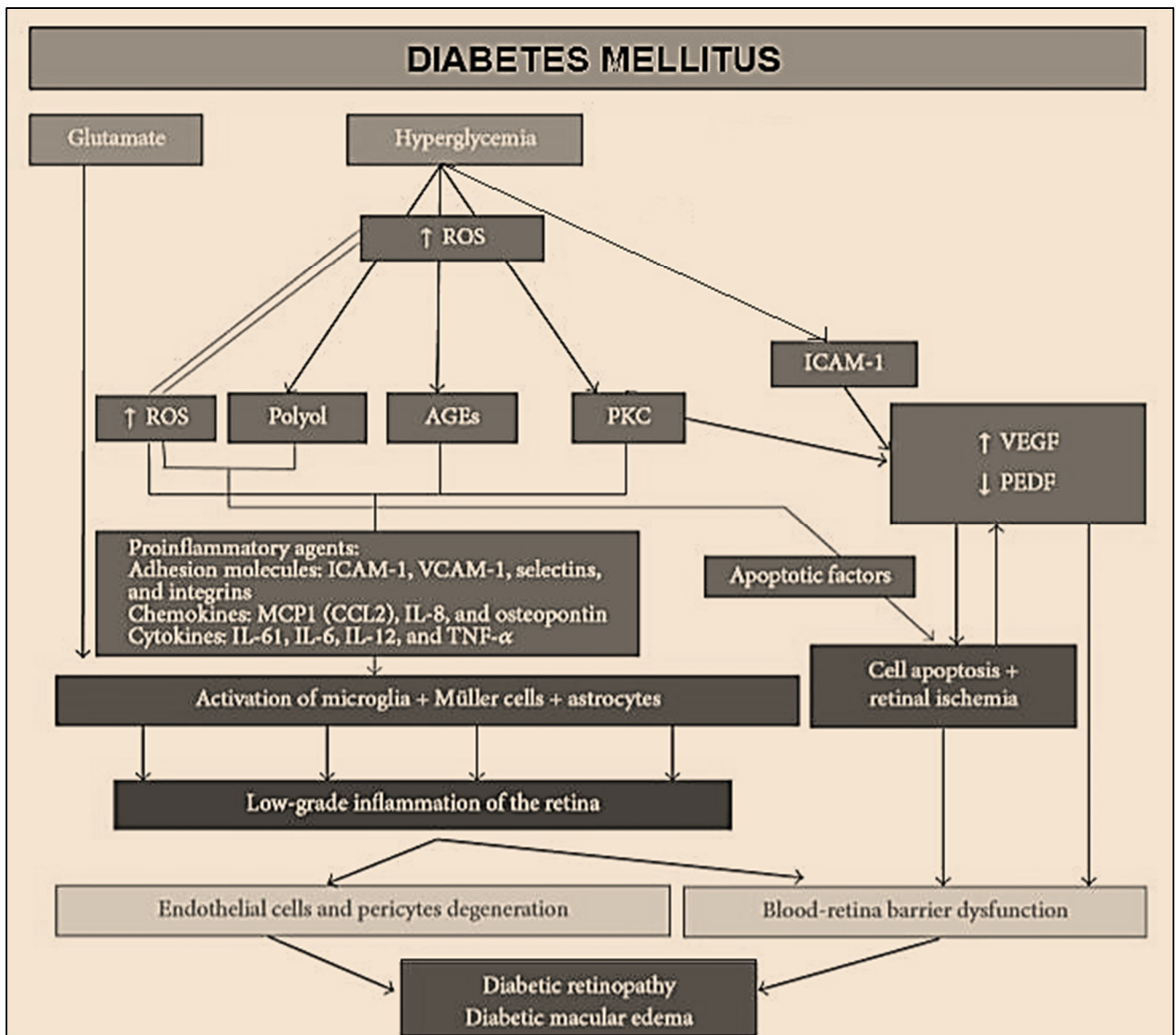
Fig. 10 Interconnected pathways involved in diabetic retinopathy

Box 4 Biochemical pathways
The first discovered mechanism was the polyol pathway and increased polyol pathway flux, described in peripheral nerves in Science in 1966 (Gabbay et al.,1966). In the late 1970s the involvement of advanced glycation end products (AGEs) was seen. In the late 1980s and early 1990s hyperglycemia-induced activation of protein kinase C (PKC) isoforms was discovered. Finally, in the late 1990s, a fourth biochemical pathway was discovered: increased hexosamine pathway flux and consequent overmodification of proteins by N-acetylglucosamine (Brownlee, 2005).

1. **Increased flux through the polyol pathway.** Aldose reductase has, physiologicaly, the function of reducing toxic aldehydes in the cell to inactive alcohols, but when the glucose concentration is too high, aldose reductase reduces and converts it into sorbitol, consuming NADPH and giving rise to a sort of hyperglycaemic pseudohypoxia; sorbitol is then oxidized into fructose. Glycation with fructose occurs

at a higher rate than with glucose alone: in diabetic patients, an excess of fructose permits the formation of a great number of advanced glycation end-products (AGEs). The production of sorbitol and the activation of the aldose-reductase enzyme, increase oxidative stress: ROS activates the poly-(ADP-ribose)-polymerase (PARP), which in turn inhibits glyceraldehyde-phosphate-dehydrogenase (GAPDH) activity, therefore pushing metabolites from glycolysis in the upstream pathways. NADPH is also the essential cofactor for regenerating a critical intracellular antioxidant, reduced glutathione. The polyol pathway decreases the production of the antioxidant glutathione, which inhibits ROS damaging retinal cells and starting DR. ROS increases the activity of protein kinase C (PKC) (**Fig 11**).
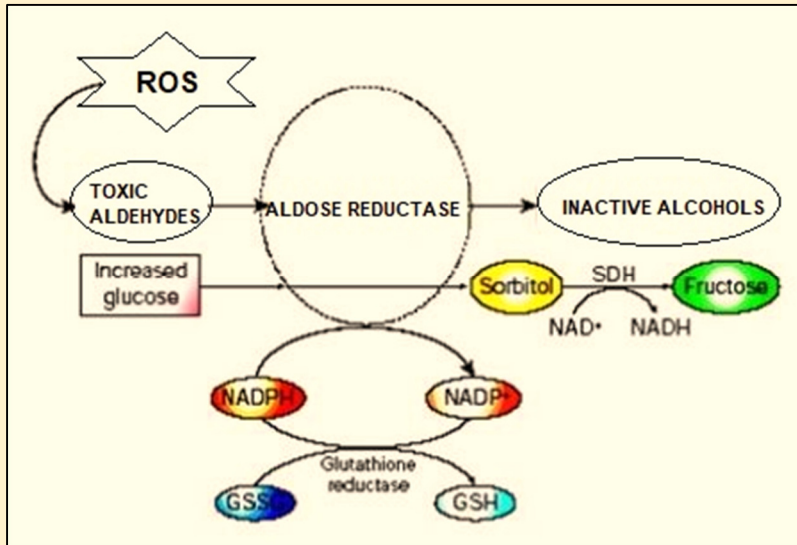

Fig. 11 Aldose reductase and the polyol pathway. Aldose reductase reduces aldehydes generated by ROS to inactive alcohols and glucose to sorbitol, using NADPH as a co-factor. In cells where aldose reductase activity is sufficient to deplete reduced glutathione (GSH), oxidative stress is augmented. Sorbitol dehydrognenase (SDH) oxidizes sorbitol to fructose using NAD$^+$ as a co-factor.

2. Increased flux through the hexosamine pathway

High glucose is metabolized through glycolysis into the cell, going first to glucose-6 phosphate, then fructose-6 phosphate, and then on through the rest of the glycolytic pathway. Some fructose-6-phosphate gets diverted into a signaling pathway in which an enzyme called GFAT (glutamine:fructose-6 phosphate amidotransferase) converts the fructose-6 phosphate to glucosamine-6 phosphate and finally to UDP (uridine diphosphate) N-acetyl glucosamine. The overmodification by this glucosamine often results in pathologic changes in gene expression (Kolm-Litty et al.,1998; Wells and Hart, 2003). It has been shown to play a role both in hyperglycemia-induced abnormalities of glomerular cell gene expression and in hyperglycemia-induced cardiomyocyte dysfunction in cell cultures. In carotid artery plaques from type 2 diabetic subjects, modification of endothelial cell proteins by the hexosamine pathway is also significantly increased.

3. Increased formation of advanced glycation end-products (AGEs)

In an early stage, sugar reacts with free amino groups inducing the glycation process and producing enzymatic glycation of proteins or advanced glycation end-products (AGEs) (irreversible compounds). An excess of AGE is one of the most important mechanisms in the pathophysiology of chronic, diabetic complications. All AGEs are highly prevalent in the retinal vasculature of diabetes patients and are involved in microvascular and macrovascular complications. (Romero-Aroca, 2016; Warboys and Fraser, 2010). AGEs stimulate the endothelial membrane receptors of advanced glycation end-products (RAGE) and it increases the expression of molecules such as inflammatory intercellular adhesion molecule-1 (ICAM-1) and VEGF, causing oxidative stress (Ono et al., 1998). The accumulation of any advanced glycation end-products (AGEs) increases the production of ROS by the mitochondria, induced by high glucose as a result of increased flux through the Krebs' cycle. AGEs increase the formation of toxic products and cell damage through their union with RAGE (AGE receptor), which can modify intracellular proteins, including those involved in the regulation of gene transcription, or diffuse outside the cell modifying the extracellular matrix, with the consequent reduction of cell-to-cell adhesion and vascular dysfunction and also modify circulating blood proteins, inducing production of inflammatory cytokines and growth factors (**Fig 12**) (Lee and Chung, 1999; Brownlee, 2001; Baynes, 1991; Giardino et al., 1994).
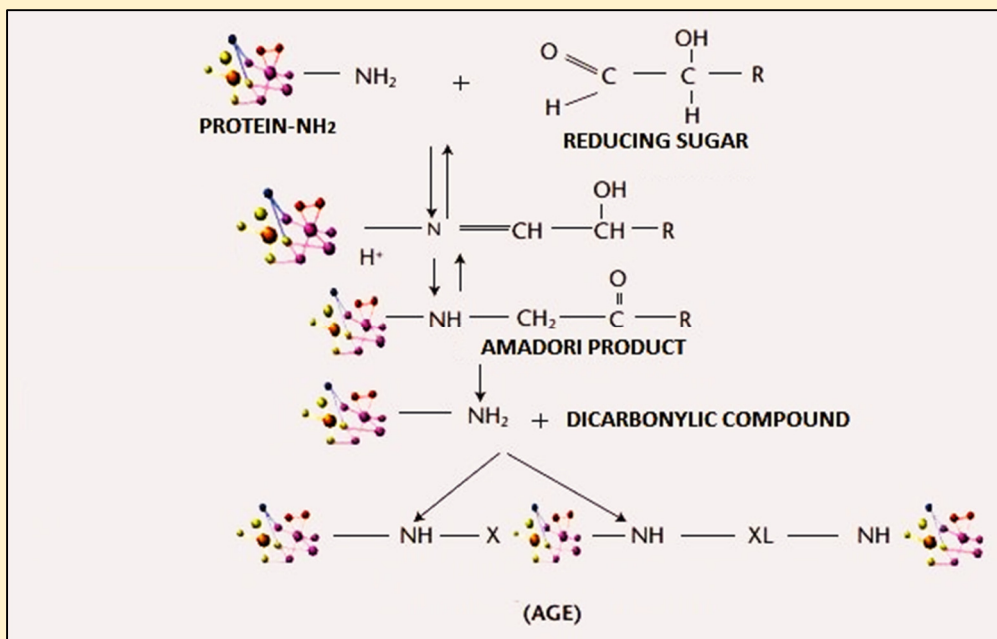

Fig. 12 Formation of advanced glycation end products in three stages. In an early stage, sugars react with a free amino group to form the Schiff base that undergoes a rearrangement to a more stable product known as the amadori product. In an intermediate stage, it degrades to a variety of reactive dicarbonyl compounds. In the late stage of the glycation process, AGEs (irreversible compounds) are formed.

4. **Activation of protein kinase C**. High glucose concentrations inside the cell also activate PKC synthesis through *de novo* excessive synthesis of the second lipid messenger diacylglycerol (DAG) which upregulates PKC activation. The activation of protein kinase C (PKC), a member of the kinase family implicated in phosphorylation reactions, is an important pathway in the disruption of internal BRB.

PKC overaction causes an upregulation of different intracellular functions, including immune-responses, cellular growth and development, vascular permeability, and angiogenesis. Other events induced directly by PKC or by increased expression of different factors such as VEGF or transforming growth factor-beta 1 (TGFβ1) include the accumulation of extracellular matrix, fibrinolysis and inflammatory responses, increasing synthesis of NF-κB (**Fig.13**).
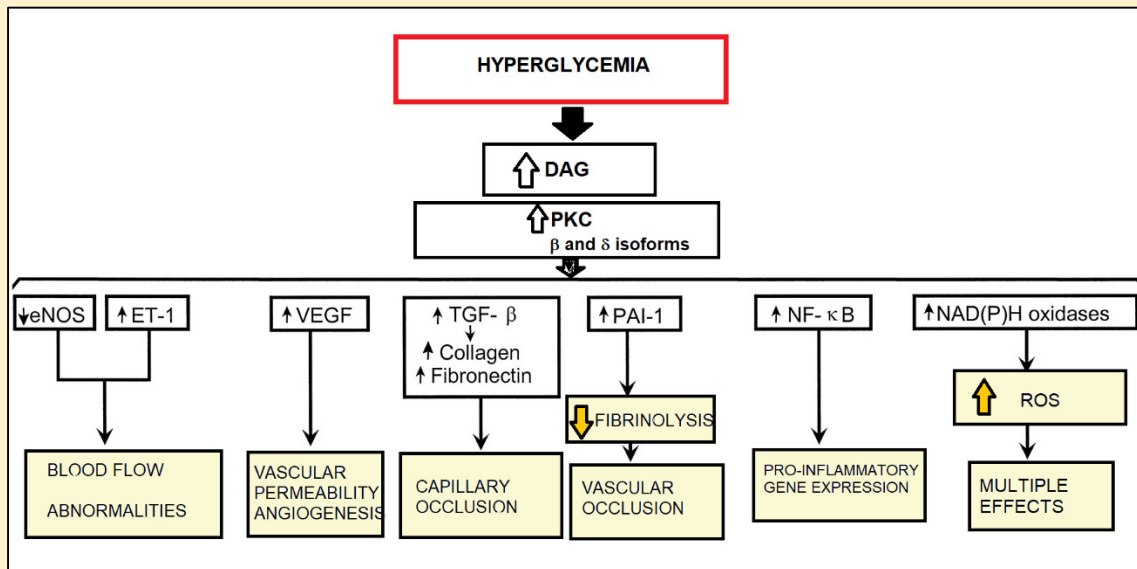

Fig. 13 The activation of protein kinase C (PKC), a member of the kinase family implicated in phosphorylation reactions, is an important pathway in the disruption of internal BRB

5   Ototoxicity

Hair cells, specialized sensory epithelia cells, can be damaged and lead to hearing loss by ototoxic drugs, including many aminoglycoside antibiotics, platinum-based anticancer agents, and diuretics, in all age groups. Box Symptoms may range from temporary tinnitus to permanent hearing loss and can be either reversible or irreversible. Ototoxic drug effects are characterized by a heterogeneous symptomatology for high inter-individual differences in genetic factors, pharmacokinetics, metabolic status of the individual and co-morbid medical conditions. In the literature there is no standardized classification of ototoxic drugs **(Box 5)**. The biological mechanisms that protect and regenerate hair cells have not been well studied, this hampers therapeutic approaches to drug-induced hearing loss (Guo et al., 2019). Ototoxicity is the pharmacological adverse reaction affecting the inner ear or auditory nerve, characterized by cochlear or vestibular dysfunction (Shi, 2016). The pathway of drug uptake from the *stria vascularis* is found to involve transporter systems as well as channels. Some ototoxic drugs, including cisplatin, cause structural damage to the *stria vascularis*. (Shi, 2016) The

intrastrial fluid-blood barrier comprises a microvascular endothelium and the accessory cells such as PCs and a specific matrix of extracellular BM, which constitute a unique "cochlear vascular unit." Disruption of the intrastrial fluid-blood barrier is considered to be involved in various clinical hearing disorders. Despite its central importance, the physiology of the intrastrial fluid-blood barrier in hearing health and disease, and the exact incidence of ototoxic side effects remain poorly understood (Shi, 2016; Bisht et al., 2011). Cisplatin may cause bilateral decreases in hearing in 61% of children (Knight et al., 2006) and between 23% and 50% in adults (Ganesan et al., 2018), the chances of incurring hearing loss is almost 63% with aminoglycosides (Bisht and Bist, 2011) and 6-7% with furosemide (Rybak, 1993). Aminoglycosides and cisplatin generate ROS in the inner ear. ROS can activate cell-death pathways such as the c-Jun Nterminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, which in turn, induce hair cell apoptosis, causing permanent hearing loss (Tabuchi et al., 2011). Gentamycin and cisplatin could cause hair cell loss or hearing loss after a single treatment. When these ototoxic drugs are administered simultaneously with high doses of loop diuretics, hair cell loss is more significant and hearing loss often develops in a permanent manner (Ding and Salvi, 2005; Liu et al., 2011). Sometimes even therapy with loop diuretics alone can lead to an adverse reaction in the cochlea, such as the formation of edematous spaces in the epithelium of the *stria vascularis*, which leads to a rapid decrease of the endolymphatic potential. Diuretic drugs interfere with strial adenylate cyclase and Na^+/K^+-ATPase and inhibit the Na-K-2Cl co-transporter in the *stria vascularis*, however, recent reports indicate that one of the earliest effects *in vivo* is to reduce blood flow in the vessels supplying the lateral wall. Since ethacrynic acid does not damage the stria vascularis *in vitro*, the changes in Na^+/K^+-ATPase and Na-K-2Cl seen *in vivo* may be secondary effects resulting from strial ischemia and anoxia. The specific location of cochlear PCs has been identified in the capillary network of the cochlear lateral wall including the vessels of the stria vascularis and spiral ligament (Shi and Xiaorui, 2011). Recent observations showing that renin is present in PCs surrounding stria arterioles suggest that diuretics may induce local vasoconstriction by renin secretion and angiotensin formation. The TJs in the blood-cochlea barrier prevent toxic molecules and pathogens from entering the cochlea, but when diuretics induce a transient ischemia, the barrier is temporarily disrupted allowing the entry of toxic chemicals or pathogens (Ding et al., 2016). Recent research shows the intrastrial fluid-blood barrier might be a main port of entry for certain ototoxic drugs, including antibacterial aminoglycoside antibiotics

such as gentamicin, anticancer agents such as cisplatin, and loop diuretics such as furosemide, from the blood into cochlear fluids and significantly increased hearing damage (Ding et al., 2012; Shi, 2016).

Box 5 Ototoxic drugs

Ototoxic drugs, including many platinum-based anticancer agents 1, aminoglycoside antibiotics 2, diuretics 3, can damaged hair cells and leaded to hearing loss, in all age groups.

1 Cisplatin

Cis-diamminedichloridoplatinum (II) (cisplatin) is a chemotherapeutic drug that has been prescribed for the treatment of many types of human cancer since its FDA approval in 1978, but its use is associated with numerous adverse effects, one of which is ototoxicity with bilateral decreases in hearing in 62% of people (Marshak et al., 2014). Children are at greater risk of cisplatin-induced ototoxicity than adults, with severe negative consequences. Cisplatin-induced ototoxicity occurs mainly in the organ of Corti that contains the sensory hair cells and supporting cells, in the *stria vascularis* that regulates the composition of the endolymph fluid, and in spiral ganglion neurons that innervate the hair cells, simultaneously. Cisplatin treatment is associated with a reduction in endocochlear potential and it is indicative of *stria vascularis* dysfunction, characterized by edema, swelling, and fewer intracellular organelles in the basal turn of the cochlea (Schacht et al., 2012). The structure of cisplatin enables it to cross the plasma membrane; it can be carried into cochlear cells by membrane transporters. In most organs cisplatin is detected within one hour after injection, and is eliminated over the following days to weeks. In contrast, the retention of cisplatin in the human cochlea persists for months to years after treatment and this drug accumulation is consistently high in the *stria vascularis*. The hyper-accumulation drives cisplatin ototoxicity (Breglio et al., 2019). The *stria vascularis* represents a likely entry point for cisplatin and the reduction in endocochlear potential by the *stria vascularis* may be the main cause of hair cell death (Breglio et al., 2019). Cisplatin-induced hearing loss is mainly attributed to oxidative stress, but recent data suggest that inflammation could contribute to inner ear cell death (Gentilin et al., 2019). Inflammatory processes may be the first adverse events induced by the drug, leading to a sequence of reactions culminating in oxidative stress and ROS production. Numerous data suggest a close connection between the release of proinflammatory cytokines, apoptosis, and ROS production in inner ear cells treated with cisplatin (Gentilin et al., 2019). Toxicity is thought to be mediated primarily through DNA crosslinking, as well as ROS production (Gale et al., 1973; Dasari and Tchounwou, 2014; Karasawa et al., 2013) that cause cell damage via lipid peroxidation, protein nitration, DNA alterations, and the amplification of inflammatory processes. Failure of the cellular defense systems against ROS-induced damage may lead to cell death, possibly involving intrinsic apoptosis, pyroptosis, endoplasmic reticulum stress, autophagy, and necroptosis (Gentilin et al., 2019). However, the pathways activated by cisplatin could be more than one. They could serve as a molecular switch for deactivating the mechanisms of action of the drug switches (**Fig. 14**)
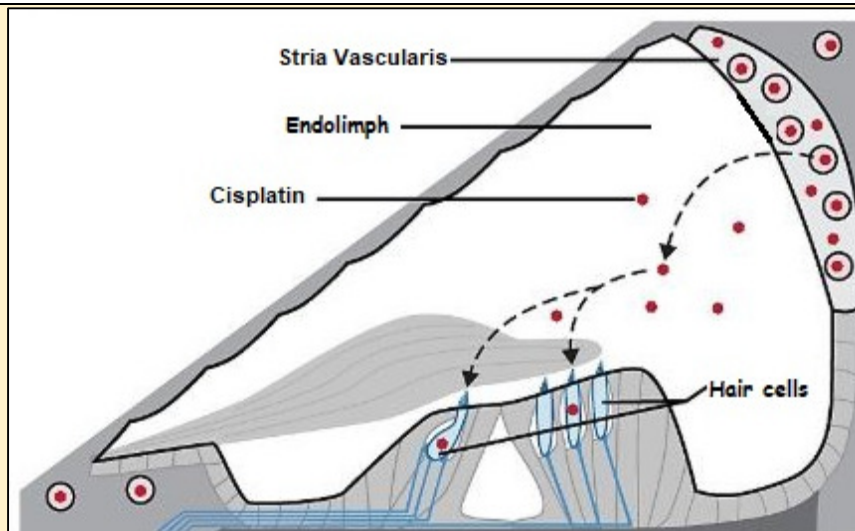
Fig. 14 Cisplatin could cross the BLB via a trans-strial trafficking route from strial capillaries, across the *stria vascularis* in the endolymph, prior to entering hair cells across their apical membranes

2 Gentamicin

Gentamicin is a broad-spectrum aminoglycoside antibiotic, isolated from Micromonospora purpurea, (Weinstein et al., 1963) which acts by binding to bacterial ribosomes and inhibiting protein synthesis. It is indicated for moderate-to-severe bacterial infections, used against many aerobic gram-negative and some aerobic gram-positive infective agents, in prophylaxis, or urgent treatment, for many life-threatening bacterial infections, including tuberculosis, sepsis, respiratory infections in cystic fibrosis, complex urinary tract infections and endocarditis. Gentamicin and other aminoglycosides are generally used in combination with a penicillin or cephalosporin for the treatment of severe infections with E. coli, Staphylococcus aureus, Enterobacter, Klebsiella, Serratia, Pseudomonas aeruginosa, and other gram-negative bacteria resistant to less toxic antibiotics. Although aminoglycosides are clinically-essential antibiotics, the nephrotoxicity and ototoxicity of these drugs are well-known and while damage inflicted on the kidney is usually reversible, damage to the inner ear is permanent (Marcotti et al., 2005). The mechanisms by which aminoglycosides enter the cochlea *in vivo* still remain poorly understood. Both endocytosis and transport through ion channels are proposed to mediate the uptake into sensory hair cells (Waguespack and Ricci, 2014): some publications describe endocytosis as preferential mechanisms of entry into hair cells, others advocate for the mechanoelectrical transducer (MET) channel located at the top of hair cell stereocilia (**Fig.15**). Vestibulotoxicity and/or cochleotoxicity occurs in many patients who receive these drugs intravenously for multiple days with a chance of incurring hearing loss of 63% (Bisht and Bist, 2011). Aminoglycosides generate free radicals within the inner ear, with subsequent permanent damage to sensory cells and neurons, resulting in permanent hearing loss. Specific factors can increase the risk of drug-induced toxicity, including sustained exposure to higher levels of ambient sound, and selected therapeutic agents such as loop diuretics and glycopeptides. Two mutations in the mitochondrial 12S ribosomal RNA gene have been previously reported to predispose carriers to aminoglycoside induced ototoxicity. Systemic aminoglycosides are predominantly and rapidly trafficked across the blood-labyrinth

barrier into the *stria vascularis*, and cleared into the endolymph prior to entering hair cells across their apical membranes. Aminoglycosides are taken up by most other cochlear cells, including fibrocytes in the lateral wall, spiral ganglion neurons, supporting cells in the organ of Corti (Selimoglu, 2007). Accumulation of gentamicin within the sensory hair cells induces apoptotic cell death when cellular protective mechanisms are overwhelmed by the toxic effects of free radicals (Alharazneh et al., 2011; Tran et al., 1986). Pharmacokinetic studies show that aminoglycoside concentrations in the endolymph follow the rise, but not the subsequent fall that is observed in serum and perilymphatic concentrations of these ototoxic drugs following systemic administration. Thus, clearance of aminoglycosides from the endolymph is very slow, and cochlear tissues retain aminoglycosides far longer than serum (Dai and Steyger, 2008).
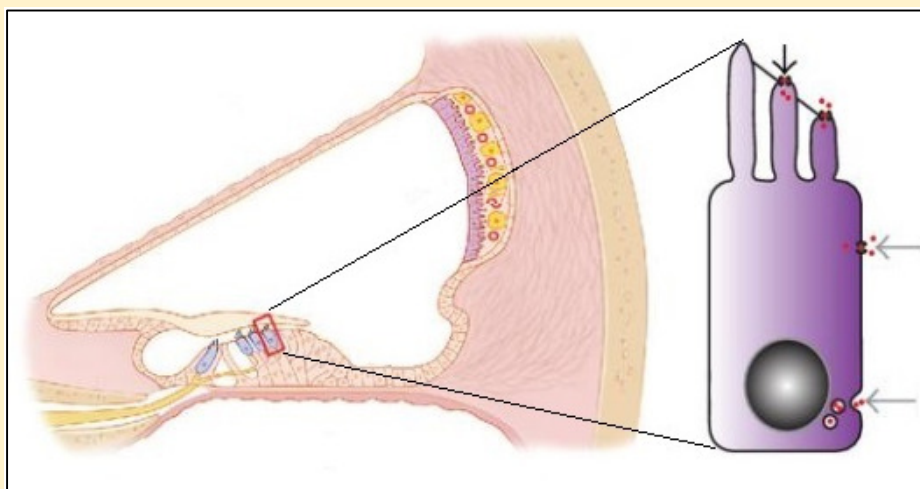

Fig. 15 Aminoglycosides can potentially enter via mechanotransducer channels located on stereocilia of hair cells or by endocytosis on the apical or basolateral membranes

3 Loop diuretics

Furosemide is a potent loop diuretic that increases the excretion of water and Na^+ by the kidneys by inhibiting their reabsorption from the proximal and distal tubules, as well as the loop of Henle. The diuretic effect is achieved through the competitive inhibition of sodium-potassium-chloride cotransporters (NKCC2) expressed along these tubules in the nephron, preventing the transport of sodium ions from the lumenal side into the basolateral side for reabsorption. This inhibition results in an increased excretion of water along with sodium, chloride, magnesium, calcium, hydrogen, and potassium ions. This drug can cause either a temporary, or in some cases, a permanent loss of hearing with vertigo in patients (Rybak, 1993), and the increased amount of available furosemide predisposes the patient to an increased risk of ototoxicity (Schellack and Alida, 2013). Loop diuretics induce unique pathological changes in the cochlea such as the formation of edematous spaces in the epithelium of the *stria vascularis*, which leads to a rapid decrease of the endolymphatic potential and eventual loss of the cochlear microphonic potential, summating potential, and compound action potential (**Fig.16**) (Rybak, 1985). The risk of loop diuretic-induced ototoxicity is greater in patients with renal impairment, in premature infants and with concomitant use of aminoglycoside antibiotics. This drug-induced loss of endocochlear potential facilitates (by unknown mechanisms) a

greater entry of aminoglycosides into the endolymph with hair cell death (Steyger et al., 2018). Animal experiments confirm that these drugs act on the *stria vascularis*, producing edema of these tissues and a temporary loss of function (Rybak, 1993). Many of the ion transporters of the stria are the same as those in the kidney, thus loop diuretics interfere with strial adenylate cyclase and Na$^+$/K$^+$-ATPase and inhibit the Na-K-2Cl cotransporter in the *stria vascularis*; these changes seen *in vivo* may be secondary effects resulting from strial ischemia and anoxia. Recent observations showing that renin is present in pericytes surrounding stria arterioles suggest that diuretics may induce local vasoconstriction by renin secretion and angiotensin formation. The TJs in the intrastrial fluid-blood barrier prevent toxic molecules and pathogens from entering the cochlea, but when diuretics induce a transient ischemia, the barrier is temporarily disrupted allowing the entry of toxic chemicals or pathogens. For this reason, simultaneous injection of loop diuretics and aminoglycoside antibiotics, e.g. gentamicin, significantly increase the peak concentration and half-life of these ototoxic drugs in the perilymph (Schellack and Alida, 2013).
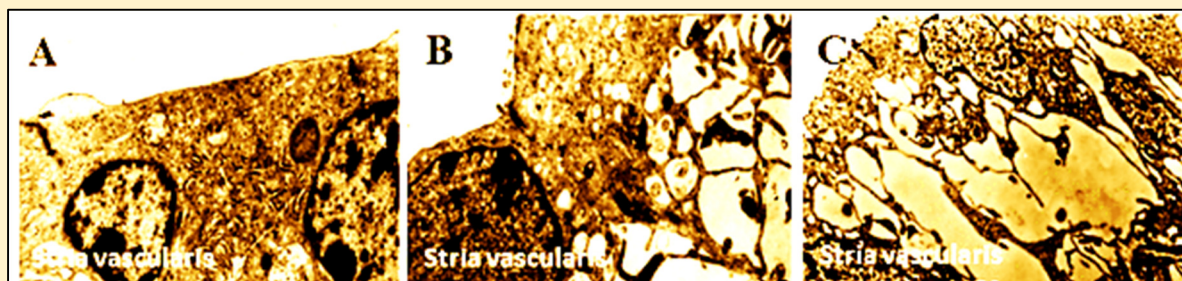

Fig 16 Epithelium of the *stria vascularis*: A in normal control animal; B strial edema present in the region of intermediate cells after short duration loop diuretic exposure; C cytoplasm of epithelium in the *stria vascularis* disrupted by edema after long duration of loop diuretic exposure

6   Aim 1

It has been seen that PCs are involved in vascular development, integrity, angiogenesis and tissue fibrogenesis. The loss of PCs contributes to the pathogenesis of many disorders such as an early feature of DR, characterised by retinal microvascular dysfunction and degeneration in the BRB (Canis and Bertlich, 2019). Since pericyte loss is virtually irreversible, only limited beneficial effects have been obtained so far by available therapeutic strategies. Therefore, every effort should be made to recover pericyte physiology and BRB integrity. ASCs are cells with regenerative, differentiation and immunomodulatory capacities (**Box 6**). Adipose tissue is also an excellent source for MSCs and the concentration of MSCs is abundant. The aim of the research was to evaluate the potential of ASCs in differentiating into the phenotype of PCs and to examine the effects of ASC like-pericytes on hRECs. The pericyte-like phenotype of the ASCs was evaluated

by immunocytochemical characterization and western blot analysis through the pericyte markers NG_2 and αSMA, compared to native PCs. Trans-wells were used to grow endothelium and pre-treated ASCs in the same medium, maintaining their separation, to evaluate the possibility to produce an *in vitro* model of the BRB. In particular, we wanted to test the effects of pericyte-like ASCs on hREC barrier formation, in comparison to hRPC induction of hREC barrier properties. Furthermore, the tightness of the barrier formed by these co-cultures was assessed by measuring TEER. Finally, three-dimensional co-cultures in Matrigel of hRECs with hRPCs or pericyte-like differentiated pericyte-like ASCs -hASCs in Matrigel were tested to investigate their behaviour.

Box 6 Adipose-Derived Mesenchymal Stem Cells

MSCs are multipotent and are present in many tissues for maintaining the homeostasis of the tissue to which they belong and in guaranteeing a certain degree of spontaneous regeneration thanks to their regenerative, differentiation and immunomodulatory capacities. hASCs are a heterogeneous group of multipotent progenitor cells that can be readily derived from adipose tissue of adult humans in very large quantities and can be taken by almost non invasive methods such as lipoaspiration (Sun et al., 2009). The studies about ASC human trials started in 2007. Unlike the cells from the the bone marrow that can only be obtained in limited quantities, adipose tissue is normally obtained in abundance. These cells are multipotent stem cells and can differentiate into adipogenic, osteogenic, chondrogenic, and myogenic cell lineages. Among the various types of MSCs, those present in adipose tissue (ASCs) have received particular interest because they have long-term self-renewal and growth capacities, a high rate of proliferation, a high yield and are able to differentiate into different cell types. For this reason, they represent an essential support for tissue regeneration (Lo Furno et al., 2018). ASCs can be found in stromal vascular fractions that are easily obtained from the dissociation of adipose tissue. ASCs have high anti-inflammation, immune regulating and angiogenic effects compared to other stem cells and compared to other clinical therapies (Sabol et al., 2018). The cells must be carefully evaluated based on stability, toxicity, contamination, and senescence in culture, furthermore, their quality depends on the purifying method and storage conditions (Neri, 2019; Palumbo et al., 2018) White adipose tissues in subcutaneous deposits is the main storage of energy, ASCs have mostly been isolated from this tissue. ASCs must express markers that are consistent with minimal criteria for defining MSCs by the International Society for Cellular Therapy (ISCT) and the International Fat Applied Technology Society. The morphology and gene expression profile of ASCs are similar to other MSCs derived from the bone marrow bone marrow and the bone marrow umbilical cord (Bourin et al., 2013; Dominici et al., 2006). ASC therapy can contribute to the promising outcome in both *in vitro* and *in vivo* experiments (**Fig. 17**).
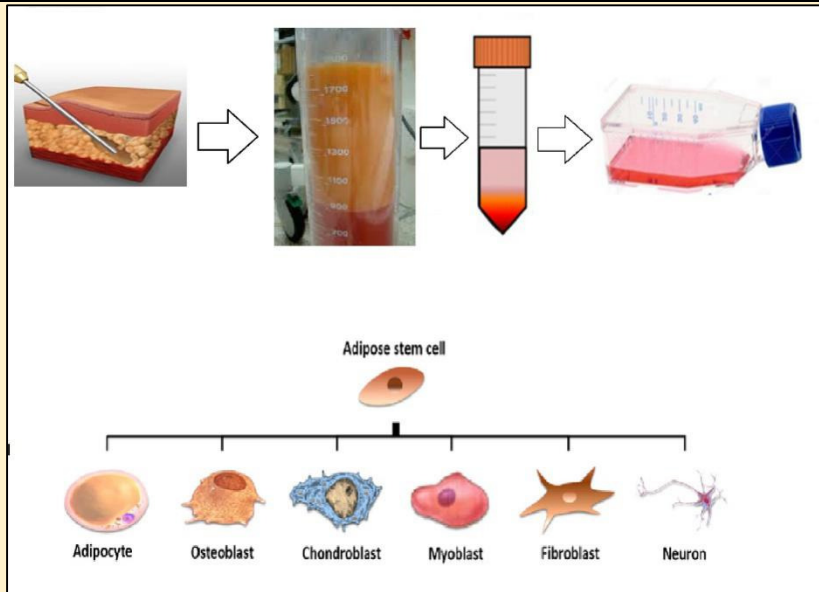
Fig. 17 Isolation of mesenchymal stem cells from adipose tissue

7   Chapter 1

Title: Pericyte-like differentiation of human adipose-derived mesenchymal stem cells: an *in vitro* study*

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Basic Study

Pericyte-like differentiation of human adipose-derived mesenchymal stem cells: an *in vitro* study

Mannino G *et al.* Pericyte-like differentiation of human ASCs

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Abstract

BACKGROUND

Adipose-derived mesenchymal stem cells (ASCs) are characterized by long-term self-renewal and high proliferation rate. Under adequate conditions, they may differentiate into cells belonging to mesodermal, endodermal or ectodermal lineages. Pericytes support endothelial cells and play an important role in stabilizing the vessel wall at the microcirculation level. The loss of pericytes, as occurs in diabetic retinopathy, results in a breakdown of the Blood-Retina Barrier (BRB) and infiltration of inflammatory cells. In this context, pericyte-like differentiated ASCs may represent a valuable therapeutic strategy for restoring a damaged BRB.

AIM

To test in vitro strategies to obtain a pericyte-like differentiation of human ASCs (hASCs).

METHODS

Different culture conditions were tested: hASCs cultured in a basal medium supplemented with Transforming Growth Factor β1 (TGF); hASCs cultured in a specific pericyte medium (PM-hASCs). In a further sample, Pericyte Growth Supplement (PGS) was omitted from PM. In addition, cultures of human Retinal Pericytes (hRPCs) were used for comparison. By immunocytochemical staining and Western Blotting, pericyte-like differentiation of hASCs was tested by the expression of α-Smooth Muscle Actin (α-SMA) and Neural/Glial antigen 2 (NG2).

Interactions between human Retinal Endothelial Cells (hRECs) and different groups of hASCs were investigated in co-culture experiments. In these cases, the expression of typical junctional proteins such as Vascular Endothelial (VE)-Cadherin, Zonula Occludens-1 (ZO-1) and Occludin were assessed in hRECs. In an in vitro model of BRB, values of the Trans Endothelial Electrical Resistance (TEER) were measured when hRECs were co-cultured with various groups of pretreated hASCs. Values observed were compared with co-cultures of hRECs and hRPCs and with cultures of hRECs alone. Three-dimensional co-cultures in Matrigel of hRECs and hRPCs or pericyte-like hASCs were designed to assess their reciprocal localization.

RESULTS

After 3-6 days of culture, α-SMA and NG2 immunocytochemistry showed that the closest pericyte-like phenotype was observed when hASCs were cultured in Pericyte Medium (PM-hASCs). In particular, α-SMA immunoreactivity, already visible at basal level in pericytes and ASCs, was strongly increased only when TGF was added to the culture medium. NG2 expression, almost undetectable in most conditions, was substantially increased only in PM-hASCs. Immunocytochemical results were confirmed by Western blot analysis. The presence of PGS seems able to improve NG2 expression (but not) rather than α-SMA increases, in agreement with its role in maintaining pericytes in the proliferative state. In co-culture experiments, immunoreactivity of VE-Cadherin, ZO-1 and Occludin was considerably increased in hRECs when hRPCs or PM-hASCs were also present. Supporting results were found by TEER measurements, gathered at 3 and 6 days of co-culture. The highest resistance values were obtained when hRECs were co-cultured with hRPCs or PM-hASCs. The pericyte-like phenotype of PM-hASCs was also confirmed in three-dimensional co-cultures in Matrigel, where PM-hASCs and hRPCs similarly localized around the tubular formations made by hRECs.

CONCLUSION

PM-hASCs seem able to strengthen the intercellular junctions between hRECs, likely reinforcing the BRB; thus, hASC-based therapeutic approaches may be usefully developed to restore the integrity of retinal microcirculation.

Mannino G, Gennuso F, Giurdanella G, Conti F, Drago F, Salomone S, Lo Furno D, Bucolo C, Giuffrida R. Pericyte-like differentiation of human adipose-derived mesenchymal stem cells: an *in vitro* study.

Core tip: Pericyte-like differentiation was achieved in hASCs by a culture medium specific for optimal pericyte growth. When co-cultured with retinal endothelial cells, pre-differentiated hASCs increased endothelial junction protein expression and trans-endothelial electrical resistance. Similar to pericytes, differentiated hASCs localized in the typical perivascular position.

INTRODUCTION

Human Adipose-derived mesenchymal Stem Cells (hASCs) have been widely studied in the last few decades for their multipotent differentiation ability. In fact, they can differentiate not only into typical mesodermal cells such as adipocytes[1], chondrocytes[2] and osteocytes[3], but also into cells of other lineages[4]. For this reason, they have been extensively investigated as a tool for potential therapeutic applications in a variety of diseases[5].

In the present investigation, the differentiation of ASCs toward a pericyte phenotype was tested *in vitro*. Pericytes share several Mesenchymal Stem Cell (MSC) features[6-8]. Both types of cells originate from the embryonic mesodermal layer, show some similar surface markers, and share some differentiation capabilities[9]. In blood vessels, pericytes surround endothelial cells and help to stabilize the vessel wall, preventing vascular leakage. Pericytes have also been associated with vessel plasticity, regression and remodeling of vascular networks[10]. Pericytes actively participate in the formation of the Blood-Retina Barrier (BRB), as they do in brain tissue, together with neurons and astrocytes, for the formation of the blood-brain barrier.

The loss of pericytes has been associated with microcirculation damage and inflammation processes[11-12], such as those occurring in Diabetic Retinopathy (DR). Loss of pericytes is one of the earliest hallmarks of DR[13-14]. As a result, the BRB is disrupted, leading to macular edema as well as new vessel formation[15]. Since pericyte loss is virtually irreversible, only limited beneficial effects have been obtained so far by available therapeutic strategies. Therefore, every effort should be made to recover pericyte physiology and BRB integrity. In the last few years, the use of stem cells has been explored to counteract DR-induced damage. Encouraging effects were reported in murine models of DR, by administration of naïve or pretreated ASCs[16]. In diabetic rodent models, beneficial effects in the stabilization of BRB breakdown were also described by Kim et al. [13], by intravitreal injections of multipotent perivascular progenitor cells derived from human embryonic stem cells.

In the present work, various culture strategies were tested in order to induce a pericyte-like differentiation of hASCs. The expression of α-Smooth Muscle Actin (α-SMA) and Neural/Glial antigen 2 (NG2) in hASCs was evaluated by immunocytochemistry and western blot analysis. and compared to that of native pericytes. In further steps, co-cultures of human Retinal Endothelial Cells (hRECs) with variously pretreated hASCs or human Retinal Pericytes (hRPCs) were also prepared to evaluate their interactions. Previous studies showed that a pericyte coverage of microvessels enhances the expression of junction proteins in endothelial cells and decreases vascular leakage[17]. Therefore, the expression

of Vascular Endothelial (VE) – cadherin, Zonula Occludens (ZO)-1 and Occludin were assessed in an *in vitro* equivalent of the BRB, comparing co-cultures of hRECs and hASCs to co-cultures of hRECs and hRPCs. Furthermore, the tightness of the barrier formed by these co-cultures was assessed by measuring the Trans-Endothelial Electrical Resistance (TEER). Finally, three-dimensional co-cultures of hRECs with hRPCs or pericyte-like differentiated hASCs in Matrigel were evaluated. A successful pericyte-like differentiation *in vitro* may be further developed in *in vivo* experiments for potential therapeutic approaches aimed at restoring the integrity of retinal microcirculation.

MATERIALS AND METHODS

hASC cultures

Adipose tissue was harvested from healthy young donors undergoing liposuction procedures at the Cannizzaro Hospital, Catania (Italy). Lipoaspirate was obtained after donors signed an informed consent to use the lipoaspirate for experimental procedures, in accordance with the Declaration of Helsinki (2000). The protocol was approved by the local ethics committee (Comitato etico Catania1; Authorization n. 155/2018/PO).

The raw lipoaspirate (50–100 ml) was washed with sterile Phosphate-Buffered Saline (PBS; Invitrogen, Monza, Italy) to remove red blood cells and debris, and incubated for 3 h at 37 °C with an equal volume of serum-free low-glucose Dulbecco's Modified Eagle Medium (DMEM-1g; Sigma-Aldrich, Milan, Italy) containing 0.075% type I collagenase (Invitrogen). After inactivation of collagenase activity by adding an equal volume of DMEM-lg containing 10% of heat-inactivated Fetal Bovine Serum (FBS, Gibco, Monza, Italy), the digested lipoaspirate was centrifuged at 1,200 rpm for 10 min. The pellets were then resuspended in PBS and filtered through a 100 µm nylon cell strainer (Falcon BD Biosciences, Milan, Italy). After a further centrifugation (1,200 rpm for 10 min), cells were plated in T75 culture flasks (Falcon BD Biosciences) with DMEM-1g containing 10% FBS, 1% penicillin/streptomycin, and 1% MSC Growth Supplement (MSCGS; ScienCell Research Laboratories, Milan, Italy). After 24 h incubation at 37 °C with 5% CO_2, non-adherent cells were removed by replacing the growth medium. After reaching confluence (about 80% of total flask surface), all cultures were trypsinized and, after resuspension, cells were expanded for 2-3 passages and plated for the following procedures.

Some cell samples were used to verify their MSC nature, according to procedures previously described[18]. In particular, their immunopositivity for typical MSC markers (CD44, CD73,

CD90 and CD105) was confirmed by immunocytochemistry and flow cytometry. At the same time, their immuno-negativity for typical hematopoietic stem cell markers (CD14, CD34 and CD45) was verified.

For the purpose of the present investigation, several groups of cultures were prepared: the first group served as control, consisting of hASCs kept in basal growth medium containing 5% FBS; in the second group, Transforming Growth Factor β1 (TGF; R & D Systems, Minneapolis, MN, USA) was added to simulate physiological conditions, where TGF-β is released by endothelial cells (TGF-hASCs); in the third group, hASCs were cultured in Pericyte Medium (PM; Innoprot, Elexalde, Derio, Spain) instead of the basal growth medium (PM-hASCs); in the fourth group, hASCs were cultured in PM, in which Pericyte Growth Supplement (PGS) was omitted (*no*PGS-hASCs). PGS (Innoprot) is a medium supplement containing growth factors, hormones, and proteins, specifically designed for the optimal growth of human pericytes *in vitro*. When added to PM, each ml of the final solution contains Bovine Serum Albumin (BSA; 10 μg), apo-transferrin (10 μg), insulin (5 mg), EGF-2 (2 ng), FGF-2 (2ng), IGF-I (2 ng) and hydrocortisone (1 mg).

From each group, some samples were stopped at 1 day, others at 3 or 6 days of culture. These samples were assessed for α-SMA and NG2 expression, by immunocytochemical staining. Other samples stopped at 3 days were processed for western blotting. Further samples of each group were trypsinized after 3 days of growth and used to prepare co-cultures with hRECs.

hRPC cultures

hRPCs were purchased from Innoprot. hRPCs were cryopreserved at secondary culture after purification and delivered frozen. hRPCs were seeded in poly-L-lysine (0.01 mg/ml solution, Innoprot) coated culture flasks and incubated at 37 °C with 5% CO_2 in PM, containing 2% FBS, 1% PGS, and 1% penicillin/streptomycin (Innoprot). At about 70% confluence, cells were trypsinized and plated for comparison with hASCs cultured in the different conditions. For this purpose, some samples were immunostained for α-SMA and NG2 at 1, 3 and 6 days of culture. Other samples, stopped at 3 days, were processed for western blot analysis. Additional samples, at 3 days of growth, were used to prepare co-culture with hRECs.

hREC cultures

hRECs, already characterized by their immunopositivity for von Willebrand factor and CD31, were purchased from Innoprot. They were seeded in culture flasks and incubated at 37 °C with 5% CO_2 in Endothelial Cell Medium (ECM, Innoprot), containing 5% FBS, 1% endothelial cell supplement factor, and 1% penicillin/streptomycin (Innoprot). When cells reached about 70% confluence, they were trypsinized and plated for co-culture experiments.

Co-cultures of hRECs with hRPCs or pretreated hASCs

After hRECs were cultured for 3 days in multiwell plates, different cell populations were added to prepare the following groups of co-cultures:

1) hRECs with untreated hASCs; 2) hRECs with TGF-hASCs; 3) hRECs with PM-hASCs; 4) hRECs with noPGS-hASCs; 5) hRECs with hRPCs.

The first group was cultured in a mixed medium containing 50% ECM and 50% basal growth medium for hASCs. A combination of 50% ECM and 50% PM was used for the other groups. In addition, a monoculture of only hRECs was used as a control.

From each group, some samples were processed after 1 day and some others after 4 days of growth. A double immune-labeling procedure was carried out to reveal simultaneously α-SMA and some typical junctional proteins, as follows: a) α-SMA and Vascular Endothelial (VE) – cadherin; b) α-SMA and Zonula Occludens (ZO)-1; c) α-SMA and Occludin.

Indirect transwell insert co-cultures

To further evaluate reciprocal interactions between hRECs and hPRCs or pretreated hASCs, a BRB-like model was set up in vitro using transwell inserts (Falcon Permeable transparent PET membrane inserts for 12-well Plate, with pore size of 0.4 µm) in which hRECs were plated on the underside and hPRCs or pretreated hASCs were seeded on the topside[19].

hRECs were first plated on the underside of the transwell inserts with a density of 15,000 cells/well. After three h, the supports were turned upside down in the culture plate. After 5 days, hPRCs (10,000 cells/well) or pretreated hASCs (10,000 cells/well) were added to the topside of the transwell inserts, opposite to hRECs. These cultures were grown in mixed media as previously described (50% ECM and 50% PM). An additional sample, containing only hRECs, seeded on the underside, was used as a reference. These indirect co-cultures were used to evaluate the Trans Endothelial Electrical Resistance (TEER) and hREC mRNA levels of junctional proteins by quantitative RT-PCR (qRT-PCR).

Immunostaining

Immunocytochemical staining was carried out following the same procedures previously described[18]. Cells were washed with PBS and fixed. Fixation with 4% paraformaldehyde for 20 min was carried out for hASC and hRPC monocultures. Fixation at -20 °C with acetone (15 min) and methanol (20 min) was preferred in co-culture experiments, when hRECs were also present. In fact, junctional proteins were better detectable after this fixation method. In the following step, cells were incubated for 30 min with a 5% solution of normal goat serum (Sigma-Aldrich) in PBS containing 0.1% Triton (Sigma-Aldrich). They were then exposed overnight at 4 °C to primary antibodies: mouse anti α-SMA (1:200; Dako M0851, Milan, Italy); rabbit anti NG2 (1:200; Abcam ab129051, Milan, Italy); rabbit anti VE-Cadherin (1:400; Cell Signaling Tecnology, D87F2, Danvers, MA, USA); rabbit anti ZO-1 (1:100; Invitrogen 61-7300); rabbit anti Occludin (1:100; Abcam ab31721).

The following day, cells were washed with PBS and incubated for 60 min at room temperature with secondary antibodies conjugated to different fluorochromes: FITC-conjugated goat anti-rabbit (1:500; Abcam) and/or Cy3-conjugated goat anti-mouse (1:500; Abcam). The specificity of immunostaining was verified in control experiments by omitting the primary antibody. Finally, DAPI staining was used to visualize cell nuclei (10 min).

Western blot analysis

Immunoblots were carried out on samples of each monoculture group (hASCs, TGF-hASCs, PM-hASCs, *no*PGS-hASCs and hRPCs), after three days of growth. Cells were trypsinized, homogenized and sonicated in RIPA buffer (Life Technologies, Monza, Italy), in the presence of a protease inhibitor cocktail (Sigma P2714), serine/threonine phosphatase inhibitors (Sigma P0044) and tyrosine protein phosphatase inhibitors (Sigma P5726). Protein concentrations were determined by BCA protein assay using BSA as standard. Cell lysates (40 μg protein) were loaded into SDS-PAGE, blotted and probed for different target proteins.

Membranes were incubated overnight at 4 °C with primary antibodies: mouse anti αSMA (1:500, Dako M0851); rabbit anti NG2 (1:500, Abcam ab 129051). The following day, membranes were incubated with secondary fluorescent antibodies (1:15,000) for 1 h at room temperature, and the immunocomplexes were detected by the Odyssey imaging system (LI-COR, Lincoln, NE). All blots were checked for equal loading by probing with β-actin antibody

(rabbit, 1:700; Sigma A2066). Densitometric analysis was performed using free software Image J (NIH, Bethesda, MD USA).

Quantitative RT-PCR

After 4 days of co-culture in transwell inserts, qRT-PCR was used to determine hREC mRNA levels of VE-Cadherin, ZO-1 and Occludin genes. Briefly, total cellular RNA was extracted from hRECs of each co-culture group using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and redissolved in 30 µl of RNase-free water. RNA concentrations and purity were estimated by optical density at 260 and 280 nm. Reverse transcription of RNA (2 µg) into first-strand cDNA was accomplished by using 200 U of SuperScript III in a 20 µl reaction volume with 50 ng random hexamers, 1.25 mM dNTP, 10 mM dithiothreitol, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ (Invitrogen). The reaction of cDNA synthesis was carried out at 50 °C for 50 min and subsequently stopped raising the temperature at 85 °C for 5 min. Aliquot of cDNA (50 ng) were amplified by employing iTaq Universal SYBR Green Supermix (Biorad, Milan, Italy) in a final volume of 20 µl (0.5 µM primers, 1.6 mM Mg^{2+}, 1X SYBR Green). Specific primers were used to amplify VE-Cadherin (forward: 5'-GGCAAGATCAAGTCAAGCGTG-3', reverse: 5'-ACGTCTCCTGTCTCTGCATCG-3'), ZO1 (forward: 5'-CAGCCGGTCACGATCTCCT-3', reverse: 5'-TCCGGAGACTGCCATTGC-3'), Occludin (forward: 5'-CACACAGGACGTGCCTTCAC-3', reverse: 5'- GAGTATGCCATGGGACTGTCAA-3') and 18S ribosomal rRNA (forward: 5′-AGTCCCTGCCCTTTGTACACA-3′, reverse: 5′-GATCCGAGGGCCTCACTAAAC-3′) purchased by Eurofins Genomics Germany GmbH. Negative controls (no template control, NTC) were included in each assay. Amplifications were carried out in a Light Cycler 1.5 instrument (Roche Diagnostics, Indianapolis, IN). The relative mRNA variations of target genes in each experimental group were calculated using ΔCT method by the comparison of the Cycle Threshold (CT) value of the gene of interest to the CT value of the selected 18S rRNA gene, considered as internal reference control gene.

Trans Endothelial Electrical Resistance (TEER)

In transwell insert co-cultures, TEER was measured daily (Ohm/cm^2) for the following 8 days with Millicell ERS-2 (Electrical Resistance System, Merck Millipore). An increase in TEER is considered as indicative of barrier formation and tightness[20]. The value of cell-free transwell insert resistance was assumed as the background resistance. Values are expressed as

Ω/cm^2 and were calculated by the following formula: (average resistance of experimental wells – average resistance of blank wells) × 0.33 (the area of the transwell membrane).

Three-dimensional co-cultures

A tube formation assay was performed in three-dimensional co-cultures *in vitro* with Matrigel Basement Membrane Matrix system (BD Discovery Labware, Bedford, MA, USA). The experimental protocol was run according to the manufacturer's instructions. Briefly, the gel solution was thawed at 4 °C overnight, then 96-well plates were coated with 50 μl of Matrigel per well and allowed to solidify at 37 °C for 2 h. A total of 15,000 cells were seeded in each well. Of these, 10,000 were hRECs and 5,000 were hRPCs or different groups of hASCs, as previously specified. Each condition was run in triplicate. After 8 h of incubation, tube-like structures were photographed by using an inverted microscope[21]. In two additional samples, hRPCs or PM-hASCs were pre-labeled with a fluorescent dye (Di-alkyl Indocarbocyanine, DiI; Invitrogen, Monza, Italy) in order to visualize their location in the co-culture.

Statistical analysis

Analysis of statistical significance was carried out by GraphPad Prism (GraphPad Software, La Jolla, CA, USA); significance between two groups was evaluated by Student's t-test. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used for multiple comparisons, p values <0.05 were considered statistically significant.

RESULTS

hASC characterization

At 24 h, hASCs cultured in the basal MSC medium exhibited a typical fibroblast-like shape. After 7 days, the cell population appeared much denser, but showed a shape similar to that observed at day 1. The stem cell profile of hASCs was verified by immunocytochemistry and flow cytometry and was analogous to that previously reported[18]; i.e., cells were immunopositive for typical MSC markers (CD44, CD73, CD90, and CD105) and immunonegative for typical hematopoietic stem cell markers (CD14, CD34, and CD45). Furthermore, according to widely accepted guidelines, they were able to differentiate into chondrocytes, adipocytes and osteocytes[3].

hASC differentiation into pericyte-like cells

Immunofluorescence

Potential differentiation of hASCs into pericyte-like cells was examined by their immunopositivity for α-SMA (Fig.1) and NG2 (fig.2), generally acknowledged as pericyte markers. Immunostaining for each marker was carried out at 1 day, 3 days and 6 days of culture. Four groups of differently treated hASCs (hASCs, TGF-hASCs, *no*PGS-hASCs, and PM-hASCs) were examined and compared to native pericytes (hRPCs). Results showed that only a basal expression of α-SMA was detectable at day 1 in hRPCs and all hASC groups (Fig.1, left column). Observations at day 3 (Fig.1, middle column) and day 6 (Fig.1, right column) showed an increased α-SMA expression in control hASCs, in TGF-hASCs, and in *no*PGS-hASCs. In these cases, a typical filamentous pattern of α-SMA organization was clearly detected. In hRPCs and PM-hASCs, the basal expression of α-SMA remained virtually unmodified (last two rows).

A clear NG2 expression was exhibited by hRPCs at day1 and was similarly detectable at day 3 and day 6 (Fig.2, last row). Among hASC groups, a similar immunoreactivity was observed only in PM-hASCs (Fig.2, second-last row), whereas NG2 immunostaining was much weaker in the other conditions.

Western blot analysis

As immunostaining modifications were already evident at day 3, this time point was chosen to further evaluate α-SMA and NG2 expression by western blot analysis (Fig. 3). These data corroborated immunofluorescence observations. In fact, histograms in Fig. 3A show that α-SMA levels present in control hASCs were similar to *no*PGS-hASCs, while much higher levels were found in TGF-hASCs; the lowest levels were observed in PM-hASCs, close to those for hRPCs.

NG2 levels (Fig. 3B) measured in control hASCs were slightly higher in TGF-hASCs and *no*PGS-hASCs; a significant increases of NG2 was observed in PM-hASCs, where it reached levels similar to hRPCs.

Co-cultures of hRECs with hRPCs or pretreated hASCs

Co-culture strategies were designed to evaluate interactions between hRECs and hASCs pre-cultured in different conditions, compared to interactions between hRECs and hRPCs. In these co-cultures, double immunostaining was carried out to visualize VE-Cadherin, ZO-1 and Occludin in HRECs, and α-SMA in the various groups of hASCs or hRPCs.

Junction protein immunostaining in hRECs

At day 1 (Fig. 4; A) and day 4 (Fig.4; B) VE-Cadherin immunostaining was carried out in hRECs, exhibiting the typical distribution at the level of the plasma membranes of adjacent cells (Fig. 4; A1, B1). VE-Cadherin expression was markedly increased when these cells had been co-cultured with hRPCs (Fig.4; A6, B6) or PM-hASCs (Fig.4; A5, B5). Lower increases were, however, also detectable in co-cultures of hRECs with the other groups of hASCs. Overall, a comparable immunostaining pattern can be observed after 1 day and 4 days of co-culture.

ZO-1 expression was already visible on plasma membranes of hRECs at day 1 (Fig. 5; A1) and day 4 (Fig. 5; B1). ZO-1 expression increased only when hRECs had been co-cultured with hRPCs (Fig. 5; A6, B6) or PM-hASCs (Fig.5; A5, B5). A similar pattern of immunostaining could be observed after 1 day and 4 days of co-culture.

Occludin immunostaining at day 1 (Fig. 6; A) and day 4 (Fig.6B) largely matched those described for VE-Cadherin and ZO-1. In fact, Occludin expression was already visible on plasma membranes of hRECs alone (Fig. 5; A1, B1), and increased when hRECs had been co-cultured with hRPCs (Fig. 6; A6, B6) or PM-hASCs (Fig. 5; A5, B5). Similar results could be observed after 1 day and 4 days of co-culture.

α-SMA immunostaining in hASCs or hRPCs

Overall, the presence of hRECs stimulated α-SMA expression in both hRPCs and hASCs, especially after 4 days of co-culture. More in detail, a strong filamentous α-SMA expression was visible at day 1 in co-cultures of hRECs and hASCs, in co-cultures of hRECs and TGF-hASCs and in co-cultures of hRECs and *no*PGS-hASCs (Figs 4-6; A2, A3, A4). In contrast, no evident α-SMA immunoreactivity was detectable in co-cultures of hRECs and PM-hASCs (Figs 4-6; A5) or in co-cultures of hRECs and hRPCs (Figs 4-6; A6). After 4 days of co-culture with hRECs, α-SMA immunoreactivity was also visible in hRPCs and PM-hASCs (Figs 4-6; B5, B6). In the other hASC groups (hASCs, TGF-hASCs and *no*PGS-hASCs), a

strong α-SMA expression was still observed (Figs 4-6; A2, A3, A4). As expected, no α-SMA immunoreactivity was detectable in cultures of hRECs alone (Figs 4-6; A1, B1).

Quantitative RT-PCR

Measurements of mRNA levels (Fig. 7) of junctional proteins in hRECs largely match results obtained by double-labelling experiments. When co-cultured with hRPCs, very high increases were observed in endothelial cells for VE-Cadherin mRNA; moderate increases were found for ZO-1, whereas no significant modifications were detected for Occludin. Increased levels of gap junction protein mRNA were also observed when hRECs were co-cultured with PM-ASCs. Compared with co-cultures with hRPCs, VE-Cadherin levels were nearly as high, whereas even higher mRNA levels were obtained for ZO-1 and Occludin. Less evident modifications were observed when hRECs were cocultured with other ASC groups.

Trans Endothelial Electrical Resistance (TEER)

Measurements of TEER values were carried out in transwell co-cultures, where a model of the BRB was set up. TEER values were considered indicative of the junctional membrane interactions between hRECs. Measurements at day 3 and 6 are reported in Fig. 8. When compared with cultures of hRECs alone, values of TEER significantly increased when hRECs had been co-cultured with hRPCs or PM-hASCs. The increase in TEER was evident at day 3 and persisted to day 6. No significant differences in TEER values were detected in the other groups of co-cultures (hRECs and hASCs, hRECs and TGF-hASCs, hRECs and *no*PGS-hASCs) when compared with hRECs alone.

Three-dimensional co-cultures (Matrigel)

Interactions between hRECs and hRPCs or different groups of hASCs were further investigated in three-dimensional co-cultures in Matrigel, where vessel-like tubular structures are spontaneously formed by endothelial cells[22]. As shown in Fig. 9, tubular structures were already visible in monoculture of hRECs after 6 h from seeding (Fig. 9; A1); the capability of forming tubular structures was maintained or improved in the presence of PM-hASCs (Fig. 9; A5) or hRPCs (Fig. 9; A6), but not in the other conditions of co-culture (Fig. 9; A2-4).

A pericyte-like phenotype of PM-hASCs was also confirmed in three-dimensional co-cultures in Matrigel after 20 h. Fig.9 B shows that in these conditions well defined tubular structures

were spontaneously assembled by hRECs alone (Fig. 9; B1). When DiI prelabeled PM-hASCs (Fig. 9; B2) or hRPCs (Fig. 9; B3) were also present, a similar localization around the tubular formations was clearly recognizable in both cases.

DISCUSSION

Data available in the literature indicate that an unequivocal immunocytochemical characterization of pericytes is hard to assess, especially because their phenotype depends on resident tissue and may change according to their activity state[9,23]. Because α-SMA and NG2 are recognized as pericyte markers[24-25], their modifications were considered indicative of a pericyte-like differentiation of hASCs. In addition, hASC immunocytochemical expression of these markers was compared to that of native pericytes (hRPCs), taken as positive control. In this way, the best culture strategy for driving hASCs to a pericyte-like phenotype was recognized. Overall, the present results indicate that a closer pericyte-like differentiation can be achieved when hASCs are grown in a culture medium specifically designed for pericytes.

Only basal levels of α-SMA were detected at early stages (1 day) of growth, both in hRPCs and hASCs, These basal levels remained unchanged in hRPCs and PM-hASCs, whereas in the other conditions (hASCs, TGF-hASCs and *no*PGS-hASCs), α-SMA expression considerably increased, especially after 3-6 days. Consistent with published data[16], the most evident increase of α-SMA expression was found when TGF-β1 had been added to the culture medium. Indeed, the increased α-SMA expression by itself is not definitely indicative of pericyte differentiation, since TGF-β1 is a strong inductor of α-SMA expression in various cell types, including pulmonary fibroblasts[26]. Therefore, a pericyte-like differentiation of hASCs was more confidently assumed by assessing the expression of NG2, which was better induced when hASCs had been cultured in PM. In this case, NG2 immunoreactivity of hASCs closely overlapped that of *bona fide* pericytes (hRPCs).

The pattern of marker expressions observed in PM-hASC was strictly related to the presence of PGS in the culture medium. In fact, when PGS had been omitted, NG2 expression was barely detectable, as in control hASCs. On the other hand, the absence of PGS provoked a marked increase of α-SMA expression. Among PGS components, Fibroblast Growth Factor (FGF) is probably responsible for these effects. In fact, it has been reported that FGF maintains cultured pericytes in a proliferative state, characterized by low levels of α-SMA[27]; consequently, the absence of FGF induces a pericyte switch from a proliferative to a contractile phenotype, where α-SMA is upregulated. Moreover, FGF is able

to antagonize TGF-β-induced α-SMA expression in pericytes and smooth muscle cells[28]. FGF-mediated proliferative effects normally decline as pericytes interact with endothelial cells, whose TGF-β production induces a switch toward the contractile phenotype[27]. It has also been reported that mesenchymal cells express smooth muscle cell markers when treated with TGF-β1 or co-cultured with endothelial cells, thus inducing precursor cells to differentiate into pericytes or smooth muscle cells[29].

We further validated the pericyte-like phenotype induced in hASCs by assessing their interaction with endothelial cells, in co-culture experiments. Compared with hRECs cultured alone, the expression of junction proteins (VE-cadherin, ZO-1 and Occludin) was more consistently increased when hRECs had been co-cultured with hRPCs or PM-hASCs, the condition that more closely matches native pericytes, rather than with hASCs cultured in other conditions. Similar conclusions may be drawn from qRT-PCR experiments. Thus, it can be assumed that these pericyte-like differentiated ASCs may mimic the functional role normally played by pericytes in strengthening the BRB. In fact, these junction proteins are mainly responsible for maintaining the tight and adherens junctions between adjacent endothelial cells[21,30-31].

In these co-cultures, it has also been observed that the presence of hRECs was able to influence α-SMA expression in hRPCs or hASCs. In particular, the typical filamentous pattern of α-SMA expression was clearly detectable in hRPCs and PM-hASCs only after 4 days of co-culture with hRECs. Instead, α-SMA immunoreactivity was poorly detectable in these two conditions after 1 day of co-culture. This was likely due to the presence of FGF in the culture medium, which induces a pericyte proliferative state. After 4 days, probably because of the endothelial secretion of growth factors such as TGF-β or Platelet Derived Growth Factor, a pericyte switching toward the contractile phenotype, characterized by a strong α-SMA expression, occurs[9,27,32]. Similar data were also reported by Rajashekhar et al. [9], showing that a significant increase of α-SMA fibrous expression occurs in ASCs located in close proximity to hRECs.

Altogether, immunocytochemical findings would suggest that PM-hASCs may represent valuable candidates for a cell-based therapy aimed at recovering a damaged BRB. In fact, when pericyte-like ASCs not yet express the filamentous α-SMA pattern, they are still in the proliferative state, the most suitable to interact with endothelial cells to build up an efficient BRB. This hypothesis is supported by TEER experiments, showing significantly increased values when hRECs had been co-cultured with hRPCs or PM-hASCs.

Supporting observations also come from experiments of three-dimensional co-cultures, where a comparable phenotype was observed for hRPCs and PM-hASCs. Both cell types tended to localize at the typical perivascular physiological position, close to the tubular structures formed *in vitro* by hRECs. Notably, this perivascular localization, together with the immunocytochemical phenotype, is considered very indicative of a pericyte-like differentiation[34]. Similar observations have been reported for bone marrow MSCs in an *in vitro* three-dimensional model of the blood brain barrier[35].

MSCs from other sources have been tested for a pericyte-like differentiation. Encouraging results have also been obtained using MSCs isolated from bone marrow and umbilical cord blood[36]. However, hASCs feature some undeniable advantages for therapeutic applications in the field of regenerative medicine. First of all, they can be harvested in large amounts from subcutaneous fat tissue, with minimal discomfort for the patient; the yield is considerably higher than that from other tissues such as the bone marrow; they feature a higher rate of proliferation and, most importantly, they are readily available for autologous treatments. Hopefully, when safe therapeutic protocols are developed, hASC-based therapeutic approaches may be successfully used to restore the integrity of a disrupted retinal microcirculation.

REFERENCES

1 **Dicker A**, Le Blanc K, Aström G, van Harmelen V, Götherström C, Blomqvist L, Arner P, Rydén M. Functional studies of mesenchymal stem cells derived from adult human adipose tissue. Exp Cell Res 2005; **308**(2): 283-290 [PMID: 15925364 DOI: 10.1016/j.yexcr.2005.04.029]

2 **Musumeci G**, Lo Furno D, Loreto C, Giuffrida R, Caggia S, Leonardi R, Cardile V. Mesenchymal stem cells from adipose tissue which have been differentiated into chondrocytes in three-dimensional culture express lubricin. Exp Biol Med (Maywood) 2011; **236**: 1333-1341 [PMID: 22036733 DOI: 10.1258/ebm.2011.011183]

3 **Calabrese G**, Giuffrida R, Lo Furno D, Parrinello NL, Forte S, Gulino R, Colarossi C, Schinocca LR, Giuffrida R, Cardile V, Memeo L. Potential effect of cd271 on human mesenchymal stromal cell proliferation and differentiation. Int J Mol Sci 2015; **16**(7): 15609-15624 [PMID: 26184166 DOI: 10.3390/ijms160715609]

4 **Lo Furno D**, Mannino G, Giuffrida R, Gili E, Vancheri C, Tarico MS, Perrotta RE, Pellitteri R. Neural differentiation of human adipose-derived mesenchymal stem cells induced by glial cell conditioned media. J Cell Physiol 2018; **233**(10): 7091-7100 [PMID: 29737535 DOI: 10.1002/jcp.26632]

5 **Lo Furno D**, Mannino G, Cardile V, Parenti R, Giuffrida R. Potential therapeutic applications of adipose-derived mesenchymal stem cells. Stem Cells Dev 2016; **25**(21): 1615-1628 [PMID: 27520311 DOI:10.1089/scd.2016.0135]

6 **Birbrair A**, Borges IDT, Gilson Sena IF, Almeida GG, da Silva Meirelles L, Gonçalves R, Mintz A, Delbono O. How plastic are pericytes? Stem Cells Dev 2017; **26**(14): 1013-1019 [PMID: 28490256 DOI: 10.1089/scd.2017.0044]

7 **Caporarello N**, D'Angeli F, Cambria MT, Candido S, Giallongo C, Salmeri M, Lombardo C, Longo A, Giurdanella G, Anfuso CD, Lupo G. Pericytes in microvessels: from "mural" function to brain and retina regeneration. Int J Mol Sci 2019; **20**(24): pii, E6351 [PMID: 31861092 DOI: 10.3390/ijms20246351]

8 **Esteves CL**, Sheldrake TA, Dawson L, Menghini T, Rink BE, Amilon K, Khan N, Péault B, Donadeu FX. Equine mesenchymal stromal cells retain a pericyte-like phenotype. Stem Cells Dev 2017; **26**(13): 964-972 [PMID: 28376684 DOI: 10.1089/scd.2017.0017]

9 **Geevarghese A**, Herman IM. Pericyte-endothelial crosstalk: implications and opportunities for advanced cellular therapies. Transl Res 2014; **163**(4): 296-306 [PMID: 24530608 DOI: 10.1016/j.trsl.2014.01.011]

10 **Berrone E**, Beltramo E, Buttiglieri S, Tarallo S, Rosso A, Hammes HP, Porta M. Establishment and characterization of a human retinal pericyte line: a novel tool for the study of diabetic retinopathy. Int J Mol Sci 2009; **23**(3): 373-378 [PMID: 19212656 DOI: 10.3892/ijmm_00000141]

11 **Platania CBM**, Giurdanella G, Di Paola L, Leggio GM, Drago F, Salomone S, Bucolo C. P2X7 receptor antagonism: implications in diabetic retinopathy. Biochem Pharmacol 2017; **138**: 130-139 [PMID: 28479300 DOI: 10.1016/j.bcp.2017.05.001]

12 **Platania CBM**, Lazzara F, Fidilio A, Fresta CG, Conti F, Giurdanella G, Leggio GM, Salomone S, Drago F, Bucolo C. Blood-retinal barrier protection against high glucose damage: the role of P2X7 receptor. Biochem Pharmacol 2019; **168**: 249-258 [PMID: 31302133 DOI: 10.1016/j.bcp.2019.07.010]

13 **Kim JM**, Hong KS, Song WK, Bae D, Hwang IK, Kim JS, Chung HM. Perivascular progenitor cells derived from human embryonic stem cells exhibit functional characteristics of pericytes and improve the retinal vasculature in a rodent model of diabetic retinopathy. Stem Cells Transl Med 2016; **5**(9): 1268-1276 [PMID: 27388242 DOI: 10.5966/sctm.2015-0342]

14 **Santos GSP**, Prazeres PHDM, Mintz A, Birbrair A. Role of pericytes in the retina. Eye (Lond) 2018; **32**(3): 483-486 [PMID: 29125148 DOI: 10.1038/eye.2017.220]

15 **Ogura S**, Kurata K, Hattori Y, Takase H, Ishiguro-Oonuma T, Hwang Y, Ahn S, Park I, Ikeda W, Kusuhara S, Fukushima Y, Nara H, Sakai H, Fujiwara T, Matsushita J, Ema M,

Hirashima M, Minami T, Shibuya M, Takakura N, Kim P, Miyata T, Ogura Y, Uemura A. Sustained inflammation after pericyte depletion induces irreversible blood-retina barrier breakdown. JCI Insight 2017; **2**(3): e90905 [PMID: 28194443 DOI: 10.1172/jci.insight.90905]

16 **Mendel TA**, Clabough EB, Kao DS, Demidova-Rice TN, Durham JT, Zotter BC, Seaman SA, Cronk SM, Rakoczy EP, Katz AJ, Herman IM, Peirce SM, Yates PA. Pericytes derived from adipose-derived stem cells protect against retinal vasculopathy. PLoS One 2013; **8**(5): e65691 [PMID: 23741506 DOI: 10.1371/journal.pone.0065691]

17 **Ting KK**, Zhao Y, Shen W, Coleman P, Yam M, Chan-Ling T, Li J, Moller T, Gillies M, Vadas MA, Gamble JR. Therapeutic regulation of VE-cadherin with a novel oligonucleotide drug for diabetic eye complications using retinopathy mouse models. Diabetologia 2019; **62**(2): 322-334 [PMID: 30443753 DOI: 10.1007/s00125-018-4770-4]

18 **Lo Furno D**, Tamburino S, Mannino G, Gili E, Lombardo G, Tarico MS, Vancheri C, Giuffrida R, Perrotta RE. Nanofat 2.0: experimental evidence for a fat grafting rich in mesenchymal stem cells. Physiol Res 2017; **66**(4): 663-671 [PMID: 28406706 DOI: 10.33549/physiolres.933451]

19 **Lupo G**, Motta C, Giurdanella G, Anfuso CD, Alberghina M, Drago F, Salomone S, Bucolo C. Role of phospholipases A2 in diabetic retinopathy: in vitro and in vivo studies. Biochem Pharmacol 2013; **86**(11): 1603-13 [PMID: 24076420 DOI: 10.1016/j.bcp.2013.09.008]

20 **Srinivasan B**, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. TEER measurement techniques for in vitro barrier model systems. J Lab Autom 2015; **20**(2): 107-26 [PMID: 25586998 DOI: 10.1177/2211068214561025]

21 **Giurdanella G**, Lazzara F, Caporarello N, Lupo G, Anfuso CD, Eandi CM, Leggio GM, Drago F, Bucolo C, Salomone S. Sulodexide prevents activation of the PLA2/COX-2/VEGF inflammatory pathway in human retinal endothelial cells by blocking the effect of AGE/RAGE. Biochem Pharmacol 2017; **142**: 145-154 [PMID: 28651842 DOI: 10.1016/j.bcp.2017.06.130]

22 **Arutyunyan IV**, Fatkhudinov TH, El'chaninov AV, Makarov AV, Kananykhina EY, Usman NY, Raimova ESh, Goldshtein DV, Bol'shakova GB. Effect of endothelial cells on angiogenic properties of multipotent stromal cells from the umbilical cord during angiogenesis modeling in the basement membrane matrix. Bull Exp Biol Med 2016; **160**(4): 575-582 [PMID: 26899836 DOI: 10.1007/s10517-016-3221-9]

23 **Schmitt BM**, Laschke MW, Rössler OG, Huang W, Scheller A, Menger MD, Ampofo E. Nerve/glial antigen (NG) 2 is a crucial regulator of intercellular adhesion molecule (ICAM)-1 expression. Biochim Biophys Acta Mol Cell Res 2018; **1865**(1): 57-66 [PMID: 28964848 DOI: 10.1016/j.bbamcr.2017.09.019]

24 **Stapor PC**, Sweat RS, Dashti DC, Betancourt AM, Murfee WL. Pericyte dynamics during angiogenesis: new insights from new identities. J Vasc Res 2014; **51**(3): 163-174 [PMID: 24853910 DOI: 10.1159/000362276]

25 **Xu J**, Gong T, Heng BC, Zhang CF. A systematic review: differentiation of stem cells into functional pericytes. FASEB J 2017; 31(5): 1775-1786 [PMID: 28119398 DOI: 10.1096/fj.201600951RRR]

26 **Vancheri C**, Gili E, Failla M, Mastruzzo C, Salinaro ET, Lo Furno D, Pistorio MP, La Rosa C, Caruso M, Crimi N. Bradykinin differentiates human lung fibroblasts to a myofibroblast phenotype via the B2 receptor. J Allergy Clin Immunol 2005; **116**(6): 1242-1248 [PMID: 16337452 DOI: 10.1016/j.jaci.2005.09.025]

27 **Papetti M**, Shujath J, Riley KN, Herman IM. FGF-2 antagonizes the TGF-beta1-mediated induction of pericyte alpha-smooth muscle actin expression: a role for myf-5 and Smad-mediated signaling pathways. Invest Ophthalmol Vis Sci 2003; **44**(11): 4994-5005 [PMID: 14578427 DOI: 10.1167/iovs.03-0291]

28 **Chen PY**, Qin L, Li G, Tellides G, Simons M. Fibroblast growth factor (FGF) signaling regulates transforming growth factor beta (TGFβ)-dependent smooth muscle cell phenotype modulation. Sci Rep 2016; **6**: 33407 [PMID: 27634335 DOI: 10.1038/srep33407]

29 **Ribatti D**, Nico B, Crivellato E. The role of pericytes in angiogenesis. Int J Dev Biol 2011; **55**(3): 261-268 [PMID: 21710434 DOI: 10.1387/ijdb.103167dr.]

30 **McGuire PG**, Rangasamy S, Maestas J, Das A. Pericyte-derived sphingosine 1-phosphate induces the expression of adhesion proteins and modulates the retinal endothelial cell barrier. Arterioscler Thromb Vasc Biol 2011; **31**(12): e107-e115 [PMID: 21940944 DOI: 10.1161/ATVBAHA.111.235408]

31 **Wisniewska-Kruk J**, Hoeben KA, Vogels IM, Gaillard PJ, Van Noorden CJ, Schlingemann RO, Klaassen I. A novel co-culture model of the blood-retinal barrier based on primary retinal endothelial cells, pericytes and astrocytes. Exp Eye Res 2012; **96**(1): 181-190 [PMID: 22200486 DOI: 10.1016/j.exer.2011.12.003]

32 **Rustenhoven J**, Aalderink M, Scotter EL, Oldfield RL, Bergin PS, Mee EW, Graham ES, Faull RL, Curtis MA, Park TI, Dragunow M. TGF-beta1 regulates human brain pericyte inflammatory processes involved in neurovasculature function. J Neuroinflammation 2016; **13**: 37 [PMID: 26867675 DOI: 10.1186/s12974-016-0503-0]

33 **Rajashekhar G**, Ramadan A, Abburi C, Callaghan B, Traktuev DO, Evans-Molina C, Maturi R, Harris A, Kern TS, March KL. Regenerative therapeutic potential of adipose stromal cells in early stage diabetic retinopathy. PLoS One 2014; **9**(1): e84671 [PMID: 24416262 DOI: 10.1371/journal.pone.0084671]

34 **Trost A**, Lange S, Schroedl F, Bruckner D, Motloch KA, Bogner B, Kaser-Eichberger A, Strohmaier C, Runge C, Aigner L, Rivera FJ, Reitsamer HA. Brain and retinal pericytes: origin, function and role. Front Cell Neurosci 2016; **10**: 20 [PMID: 26869887 DOI: 10.3389/fncel.2016.00020]

35 **Tian X**, Brookes O, Battaglia G. Pericytes from Mesenchymal Stem Cells as a model for the blood-brain barrier. Sci Rep 2017; **7**: 39676 [PMID: 28098158 DOI: 10.1038/srep39676]

36 **Salehi H**, Amirpour N, Razavi S, Esfandiari E, Zavar R. Overview of retinal differentiation potential of mesenchymal stem cells: A promising approach for retinal cell therapy. Ann Anat 2017; **210**: 52-63 [PMID: 27986614 DOI: 10.1016/j.aanat.2016.11.010]
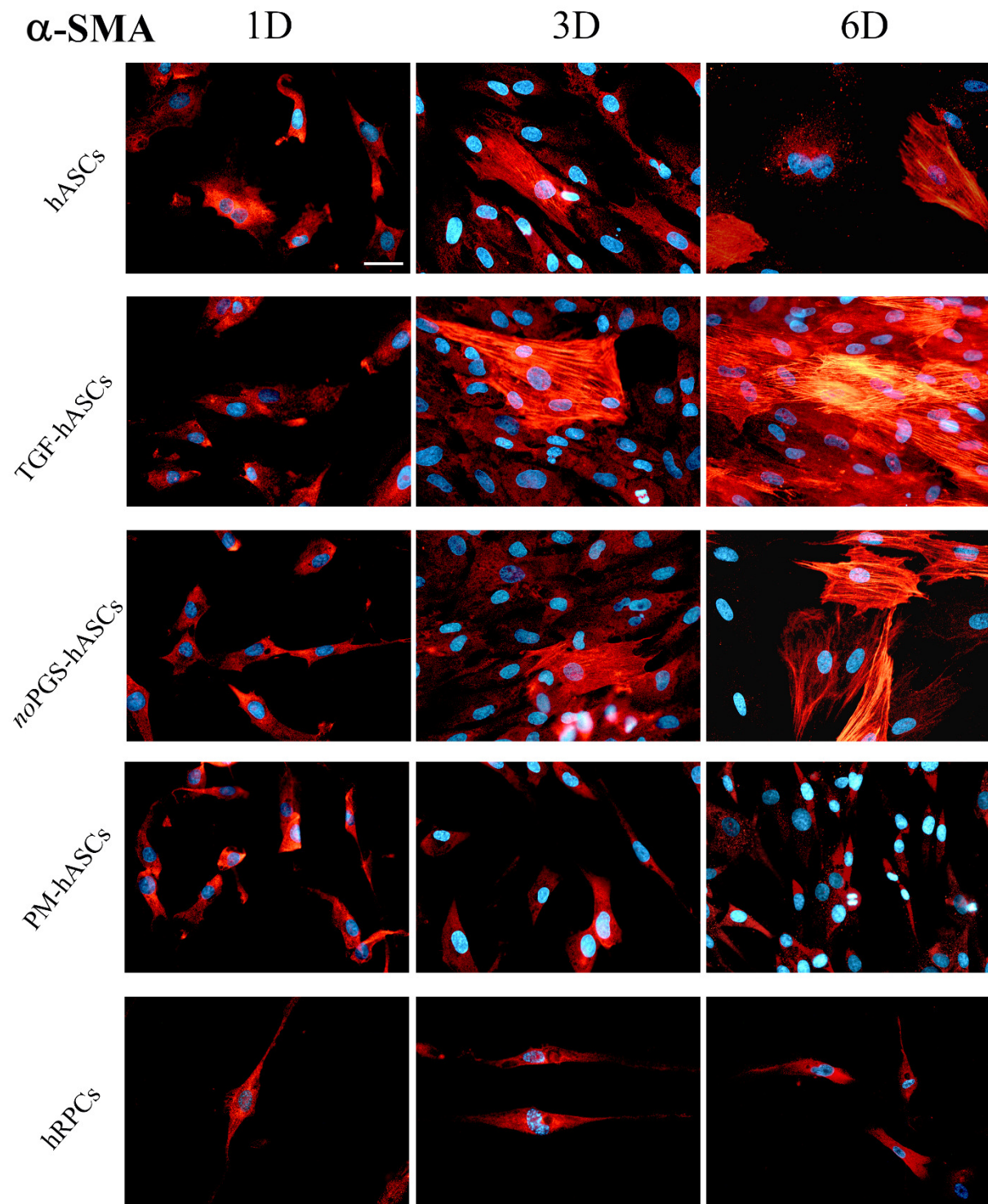
FIGURES

Figure 1 - α-Smooth Muscle Actin (α-SMA) immunoreactivity (red fluorescence) evaluated after 1 day (left column, 1D), three days (middle column, 3D) and six days (right column, 6D) of cell growth.

First row: human Adipose derived mesenchymal Stem Cells (hASCs) cultured in basal medium.

Second row: hASCs in basal medium stimulated with Transforming Growth Factor (TGF-hASCs).

Third row: hASCs cultured in Pericyte Medium lacking Pericyte Growth Supplement (*no*PGS-hASCs).

Fourth Row: hASCs cultured in complete Pericyte Medium (PM-hASCs).

Fifth row: human Retinal Pericyte Cells cultured in complete Pericyte Medium (hRPCs).

Photomicrographs show that only a basal expression of α-SMA was detectable in hRPCs and all hASC groups at day 1 (left column). At day 3 and 6, a typical filamentous pattern of α-SMA was clearly detected in hASCs, TGF-hASCs and *no*PGS-hASCs, whereas the basal expression of α-SMA remained virtually unmodified in PM-hASCs and hRPCs (last two rows). Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm.
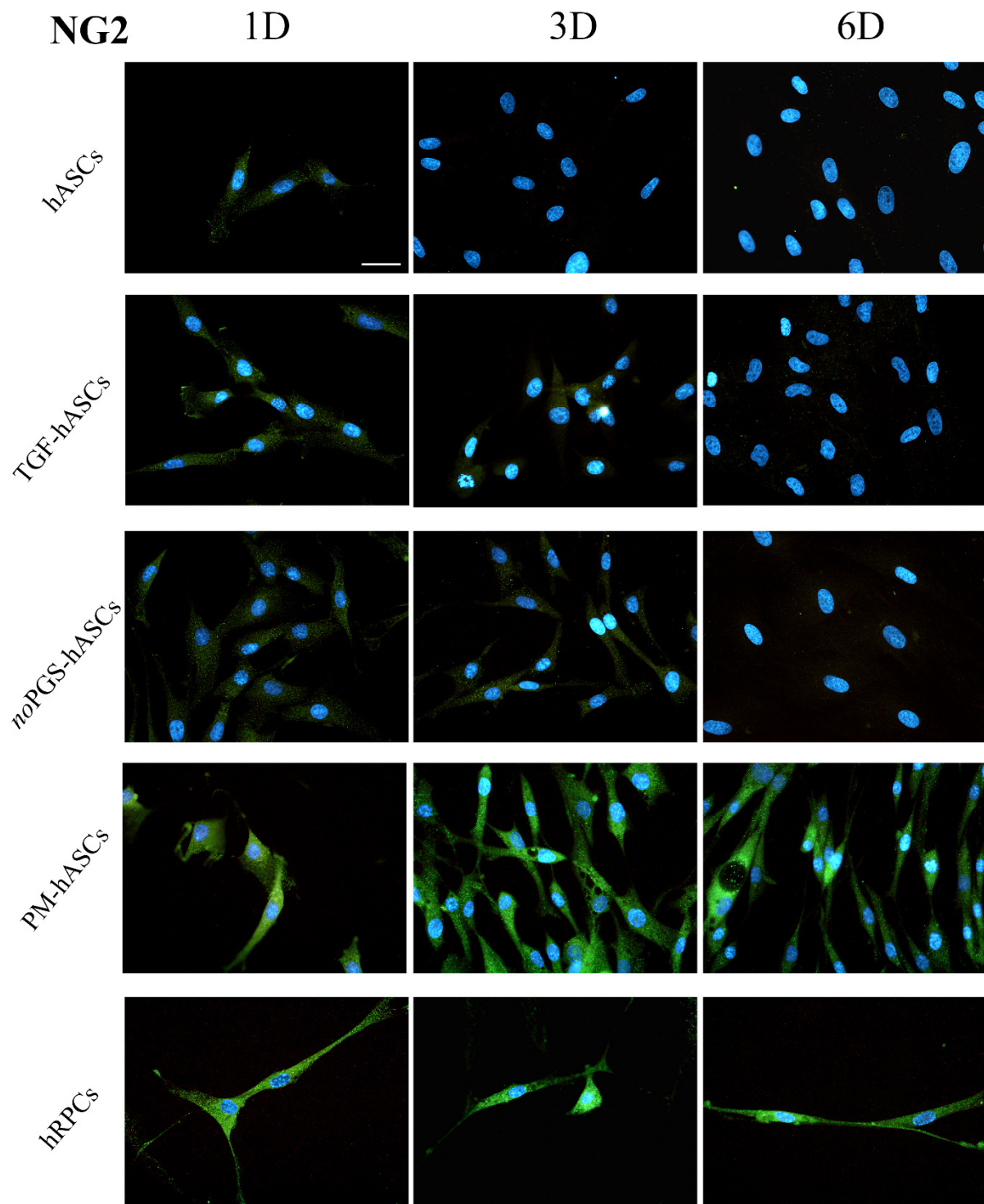
Figure 2 - Neural/Glial antigen 2 (NG2) immunoreactivity (green fluorescence) evaluated after 1 day (left column, 1D), three days (middle column, 3D) and six days (right column, 6D) of cell growth.

First row: human Adipose derived mesenchymal Stem Cells (hASCs) cultured in basal medium.

Second row: hASCs in basal medium stimulated with Transforming Growth Factor (TGF-hASCs).

Third row: hASCs cultured in Pericyte Medium lacking Pericyte Growth Supplement (*no*PGS-hASCs).

Fourth Row: hASCs cultured in complete Pericyte Medium (PM-hASCs).

Fifth row: human Retinal Pericyte Cells cultured in complete Pericyte Medium (hRPCs).

Photomicrographs show that a clear NG2 immunoreactivity is present only in PM-hASCs and hRPCs (last two rows). The immunostaining detected at day1 is similar to that observed at day 3 and 6. Very weak immunostaining is visible in the other three hASC groups (first three rows).

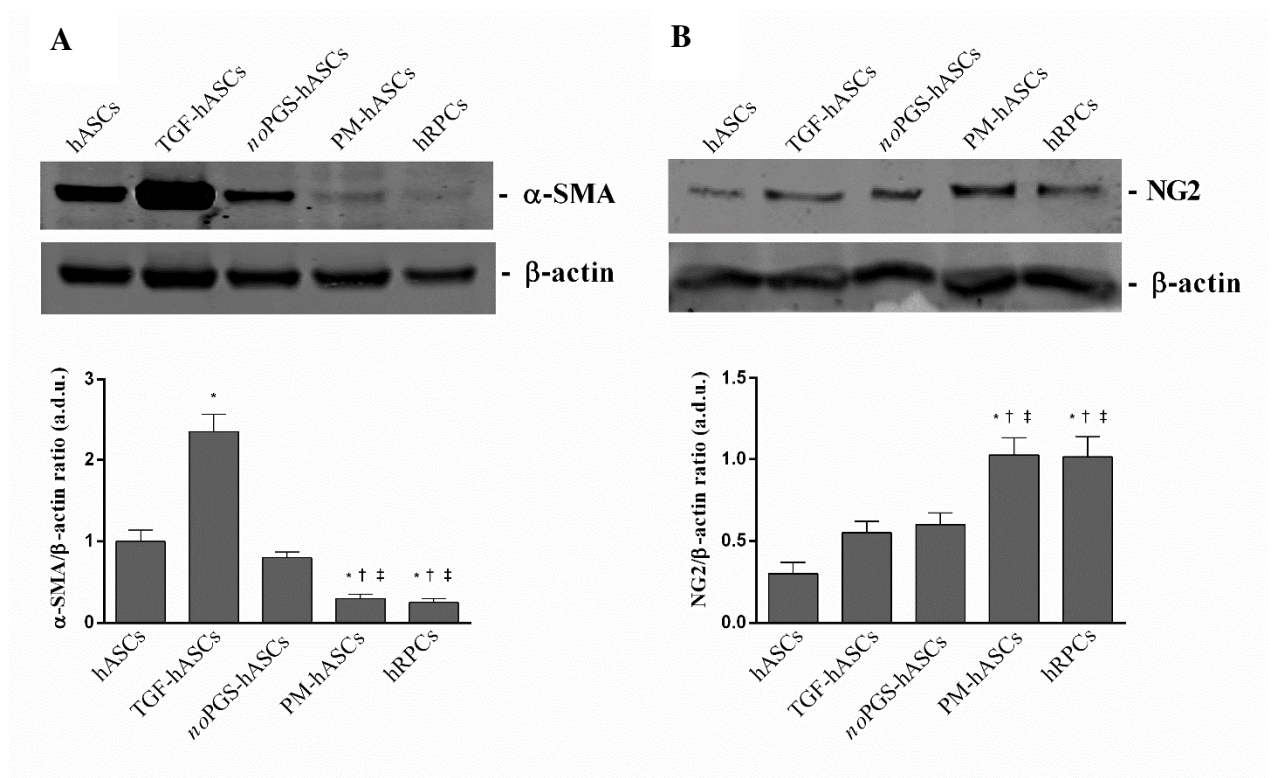Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm.

Figure 3 - Western blot analysis of α-SMA (A) and NG2 (B) expression in different groups of hASCs and hRPCs, at 3 days of culture. Histograms in A show that α-SMA levels measured in control hASCs are similar to *no*PGS-hASCs; much higher levels were found in TGF-hASCs; the lowest levels were observed in PM-hASCs, close to those for hRPCs.

Histograms in B show that NG2 levels measured in control hASCs are slightly higher in TGF-hASCs and *no*PGS-hASCs; significant increases were observed in PM-hASCs, similarly to those for hRPCs.

* indicates significant difference (p<0.05) vs hASCs;

† indicates significant difference (p<0.05) vs TGF-hASCs;

‡ indicates significant difference (p<0.05) vs *no*PGS-hASCs.

Abbreviations:

α-SMA, alpha Smooth Muscle Actin;

hASCs, human Adipose derived mesenchymal Stem Cells pre-cultured in basal medium;

hRPCs, human Retinal Pericyte Cells;

NG2, Neural/Glial antigen 2;

*no*PGS-hASCs, hASCs pre-cultured in Pericyte Medium lacking Pericyte Growth Supplement;

PM-hASCs, hASCs pre-cultured in complete Pericyte Medium;

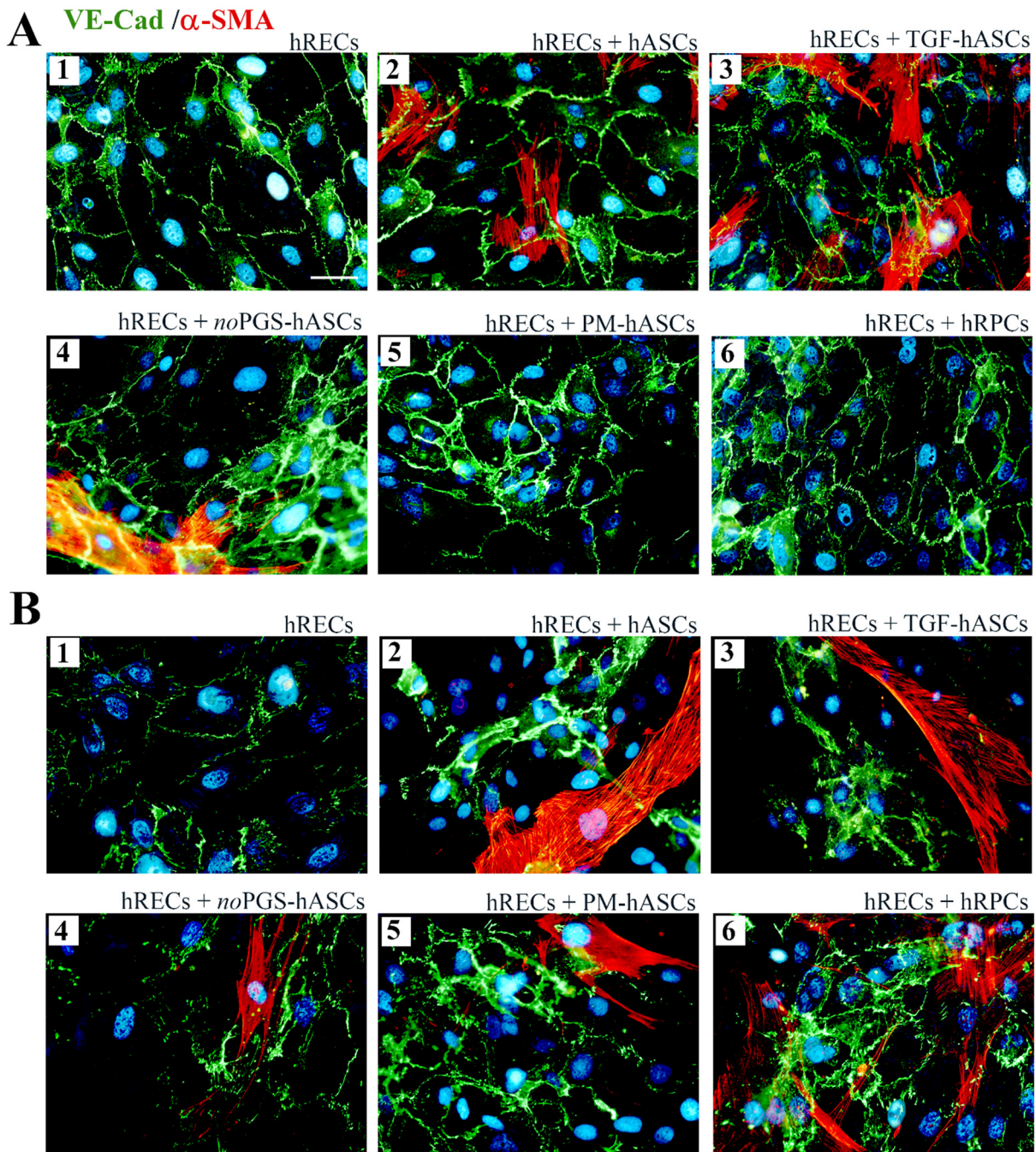TGF-hASCs, hASCs pre-stimulated with Transforming Growth Factor;

Figure 4 – Double labeling experiments of VE-Cad (green immunofluorescence) and α-SMA (red immunofluorescence) in co-cultures of hRECs and hRPCs or different groups of hASCs. Photomicrographs show results obtained after 1 day (A) and 4 days (B) of co-culture.

Photomicrographs in A1 and B1 refer to cultures of hRECs alone which are taken as reference. In these cultures, only VE-Cad immunostaining is present, showing the typical localization at the level of plasma membranes of adjacent cells.

At day 1, when hRECs are co-cultured with different groups of hASCs (A2-A5) or hRPCs (A6), VE-Cad immunostaining appears more intense. A similar trend of VE-Cad immunostaining is recognizable after 4 days of co-culture (B2-B6).

At day 1 of co-culture, an α-SMA typical filamentous pattern is visible only in hASCs pre-cultured in their basal medium (A2), pre-stimulated with TGF (A3) or pre-cultured in *no*PGS medium (A4). No α-SMA immunostaining is detected in hRPCs (A6) or in hASCs pre-cultured in PM (A5). At day 4, α-SMA immunostaining is detectable also in these two conditions (B5, B6).

No α-SMA immunoreactivity is noticeable in A1 and B1, which refer to cultures of hRECs alone. Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm.

Abbreviations:

α-SMA, alpha Smooth Muscle Actin;

hASCs, human Adipose derived mesenchymal Stem Cells pre-cultured in basal medium;

hRECs, human Retinal Endothelial Cells;

hRPCs, human Retinal Pericyte Cells;

*no*PGS-hASCs, hASCs pre-cultured in Pericyte Medium lacking Pericyte Growth Supplement;

PM-hASCs, hASCs pre-cultured in complete Pericyte Medium;

TGF-hASCs, hASCs pre-stimulated with Transforming Growth Factor;
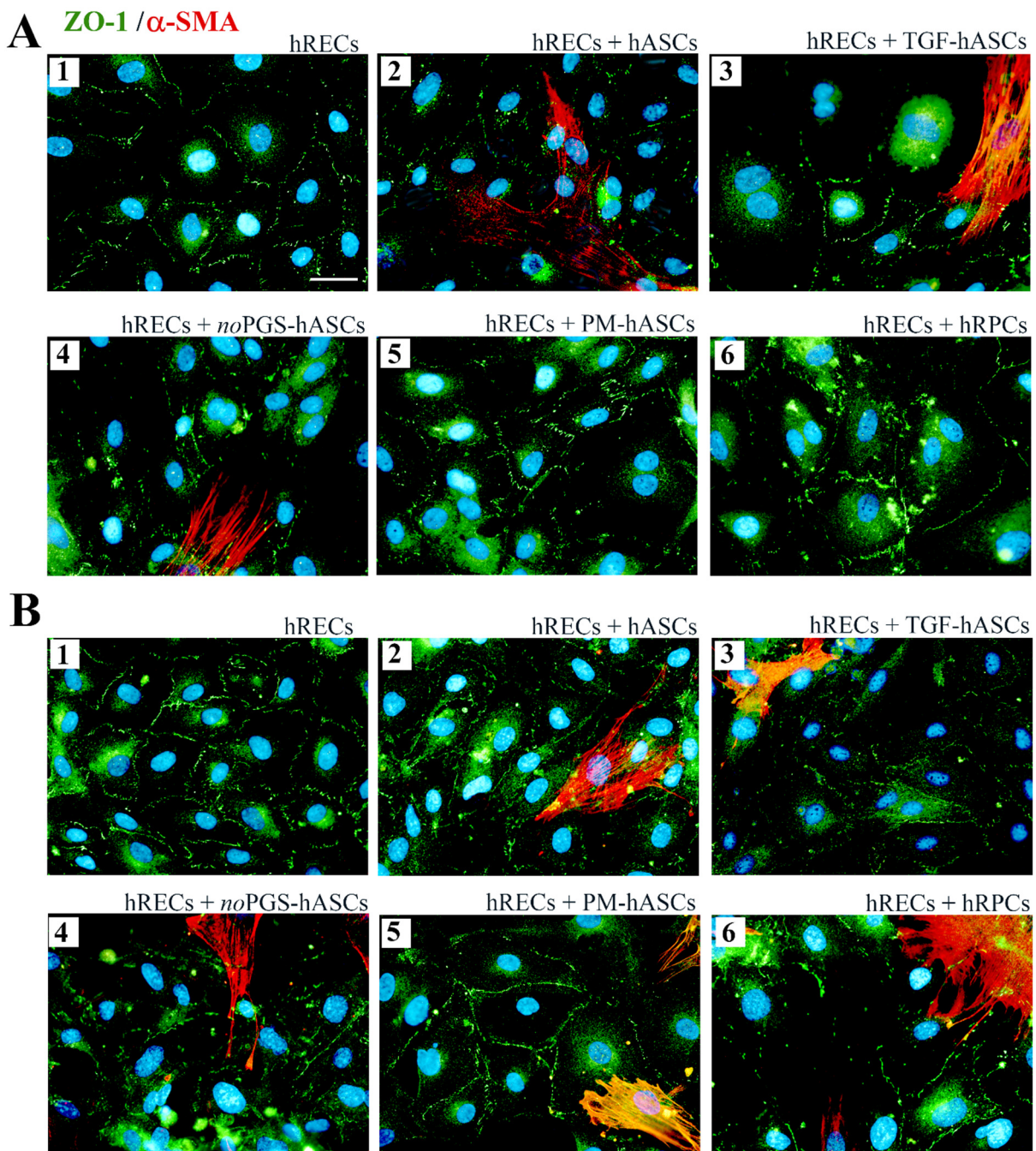
VE-Cad, Vascular Endothelial-Cadherin.

Figure 5 – Double labeling experiments of ZO-1 (green immunofluorescence) and α-SMA (red immunofluorescence) in co-cultures of hRECs and hRPCs or different groups of hASCs. Photomicrographs show results obtained after 1 day (A) and 4 days (B) of co-culture.

Photomicrographs in A1 and B1 refer to cultures of hRECs alone which are taken as reference. In these cultures, only ZO-1 immunostaining is present showing the typical localization at the level of plasma membranes of adjacent cells.

At day 1, when hRECs are co-cultured with different groups of hASCs (A2-A5) or hRPCs (A6), ZO-1 immunostaining appears more intense. A similar trend of ZO-1 immunostaining is recognizable after 4 days of co-cultures (B2-B6).

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At day 1 of co-culture, an α-SMA typical filamentous pattern is clearly detectable only in hASCs pre-cultured in their basal medium (A2), pre-stimulated with TGF (A3), or pre-cultured in *no*PGS medium (A4). Instead, no α-SMA immunostaining is detected in hRPCs (A6) or in hASCs pre-cultured in PM (A5). At 4 days, α-SMA immunostaining is detectable also in these two conditions (B5, B6).

No α-SMA immunoreactivity is noticeable in A1 and B1, which refer to cultures of hRECs alone.

Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm.

Abbreviations:

α-SMA, alpha Smooth Muscle Actin;

hASCs, human Adipose derived mesenchymal Stem Cells pre-cultured in basal medium;

hRECs, human Retinal Endothelial Cells;

hRPCs, human Retinal Pericyte Cells;

TGF-hASCs, hASCs pre-stimulated with Transforming Growth Factor;

*no*PGS-hASCs, hASCs pre-cultured in Pericyte Medium lacking Pericyte Growth Supplement;

PM-hASCs, hASCs pre-cultured in complete Pericyte Medium;
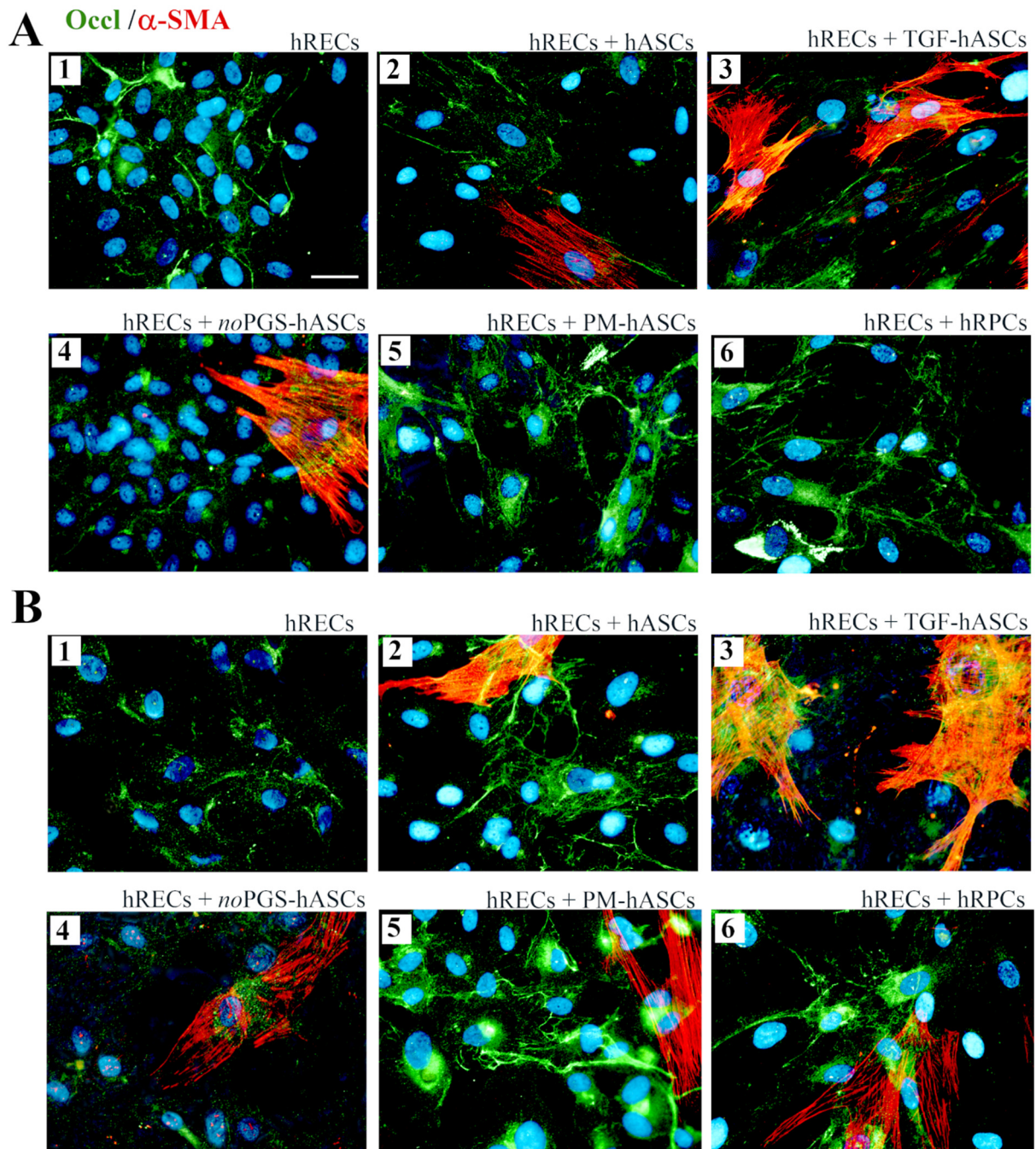
ZO-1, Zonula Occludens 1.

Figure 6 – Double labeling experiments of Occl (green immunofluorescence) and α-SMA (red immunofluorescence) in co-cultures of hRECs and hRPCs or different groups of hASCs. Photomicrographs show results obtained after 1 day (A) and 4 days (B) of co-culture.

Photomicrographs in A1 and B1 refer to cultures of hRECs alone which are taken as reference. In these cultures, only Occl immunostaining is present.

At day 1, when hRECs are co-cultured with different groups of hASCs (A2-A5) or hRPCs (A6), Occl immunostaining appears more intense. A similar trend of Occl immunostaining is recognizable after 4 days of co-cultures (B2-B6).

At day 1 of co-culture, an α-SMA typical filamentous pattern is clearly detectable only in hASCs pre-cultured in their basal medium (A2), pre-stimulated with TGF (A3), or pre-cultured in *no*PGS medium (A4). On the contrary, no α-SMA immunostaining is detected in hRPCs (A6) or in hASCs pre-cultured in PM (A5). At 4 days, α-SMA immunostaining is detectable also in these two conditions (B5, B6).

No α-SMA immunoreactivity is noticeable in A1 and B1, which refer to cultures of hRECs alone.

Blue fluorescence indicates DAPI staining of cell nuclei. Scale bar: 50 μm.

Abbreviations:

α-SMA, alpha Smooth Muscle Actin;

hASCs, human Adipose derived mesenchymal Stem Cells pre-cultured in basal medium;

hRECs, human Retinal Endothelial Cells;

hRPCs, human Retinal Pericyte Cells;

TGF-hASCs, hASCs pre-stimulated with Transforming Growth Factor;

*no*PGS-hASCs, hASCs pre-cultured in Pericyte Medium lacking Pericyte Growth Supplement;

Occl, Occludin;

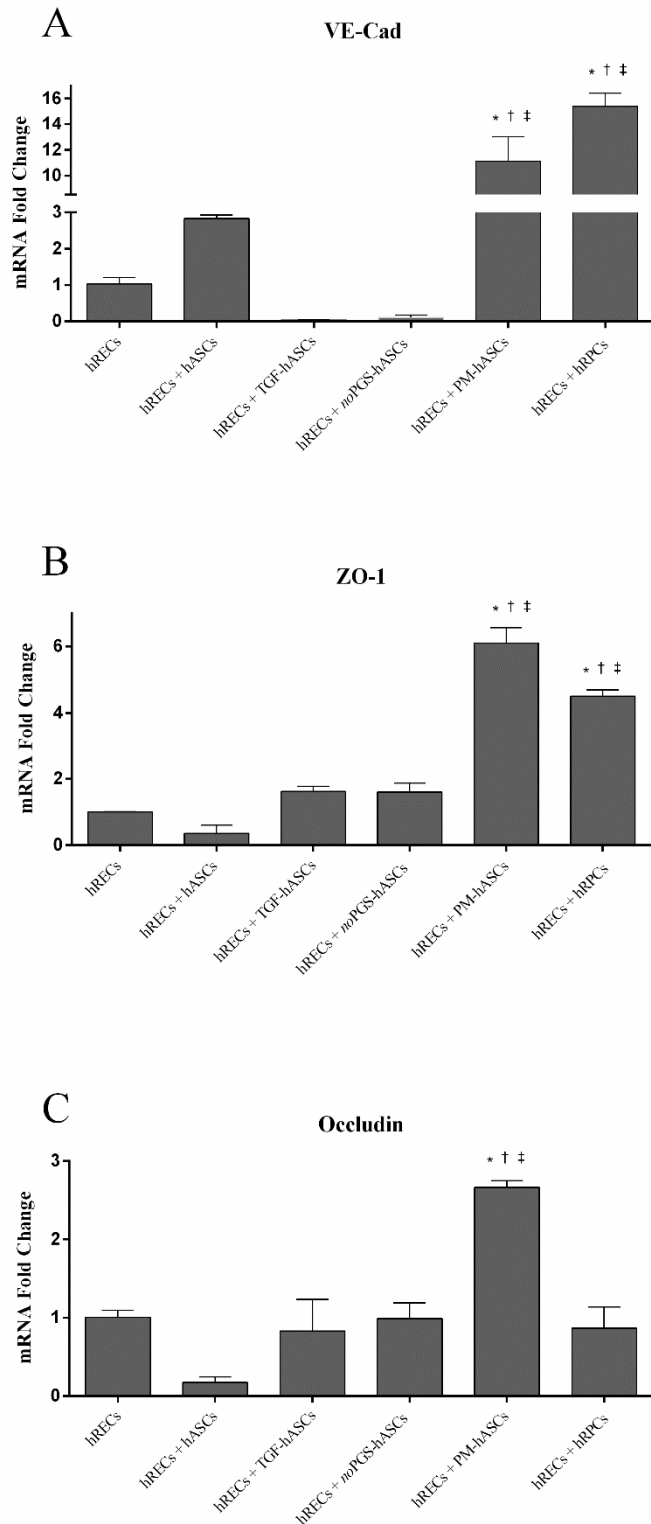PM-hASCs, hASCs pre-cultured in complete Pericyte Medium.

Fig. 7. mRNA levels of junction proteins in hRECs after 4 days of co-culture with hRPCs or different groups of hASCs. Histogram bars represent mRNA fold changes for VE-Cadherin (A), ZO-1 (B) and Occludin (C). Results are referred to the control levels of hRECs and normalized to the RNA expression of the housekeeping reference ribosomial gene 18S. All

data represent mean ± SEM obtained from at least three independent experiments. One-way ANOVA, followed by Tukey's test.

* indicates significant difference ($p < 0.05$) vs hRECs;

† indicates significant difference ($p < 0.05$) vs TGF-hASCs;

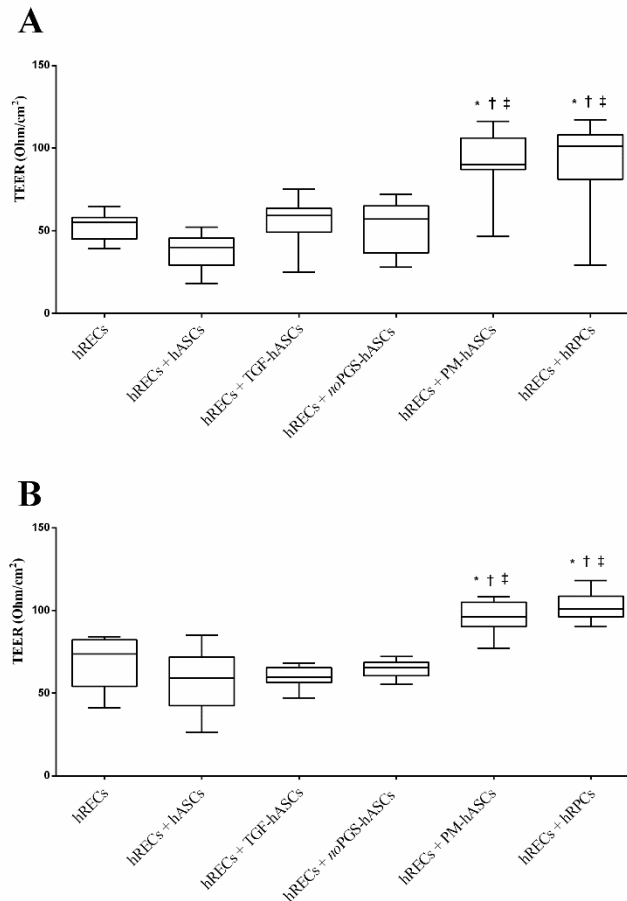‡ indicates significant difference ($p < 0.05$) vs *no*PGS-hASCs.

Figure 8 - Evaluation of the barrier integrity by Transendothelial Electrical Resistance (TEER) in hREC monocultures or in cocultures of hRECs with pericytes (hRPCs) or different groups of hASCs.

TEER values measured at day 3 (A) and day 6 (B) show that significant increases were observed only when hRECs were co-cultured with PM-hASCs or hRPCs. The boxes define the 25th and 75th percentile; the center line the median; the whiskers the range. Values are expressed as Ohm/cm^2 and calculated by the formula: (average resistance of experimental wells - average resistance of blank wells) x 0.33 (area of the transwell membrane). Values are gathered from three independent experiments carried out in duplicate (n = 6).

* indicates significant difference (p<0.05) vs hRECs;

† indicates significant difference (p<0.05) vs TGF-hASCs;

‡ indicates significant difference (p<0.05) vs noPGS-hASCs.

Abbreviations:

hASCs, human Adipose derived mesenchymal Stem Cells pre-cultured in basal medium;

hRECs, human Retinal Endothelial Cells;

hRPCs, human Retinal Pericyte Cells;

TGF-hASCs, hASCs pre-stimulated with Transforming Growth Factor;

*no*PGS-hASCs, hASCs pre-cultured in Pericyte Medium lacking Pericyte Growth Supplement;

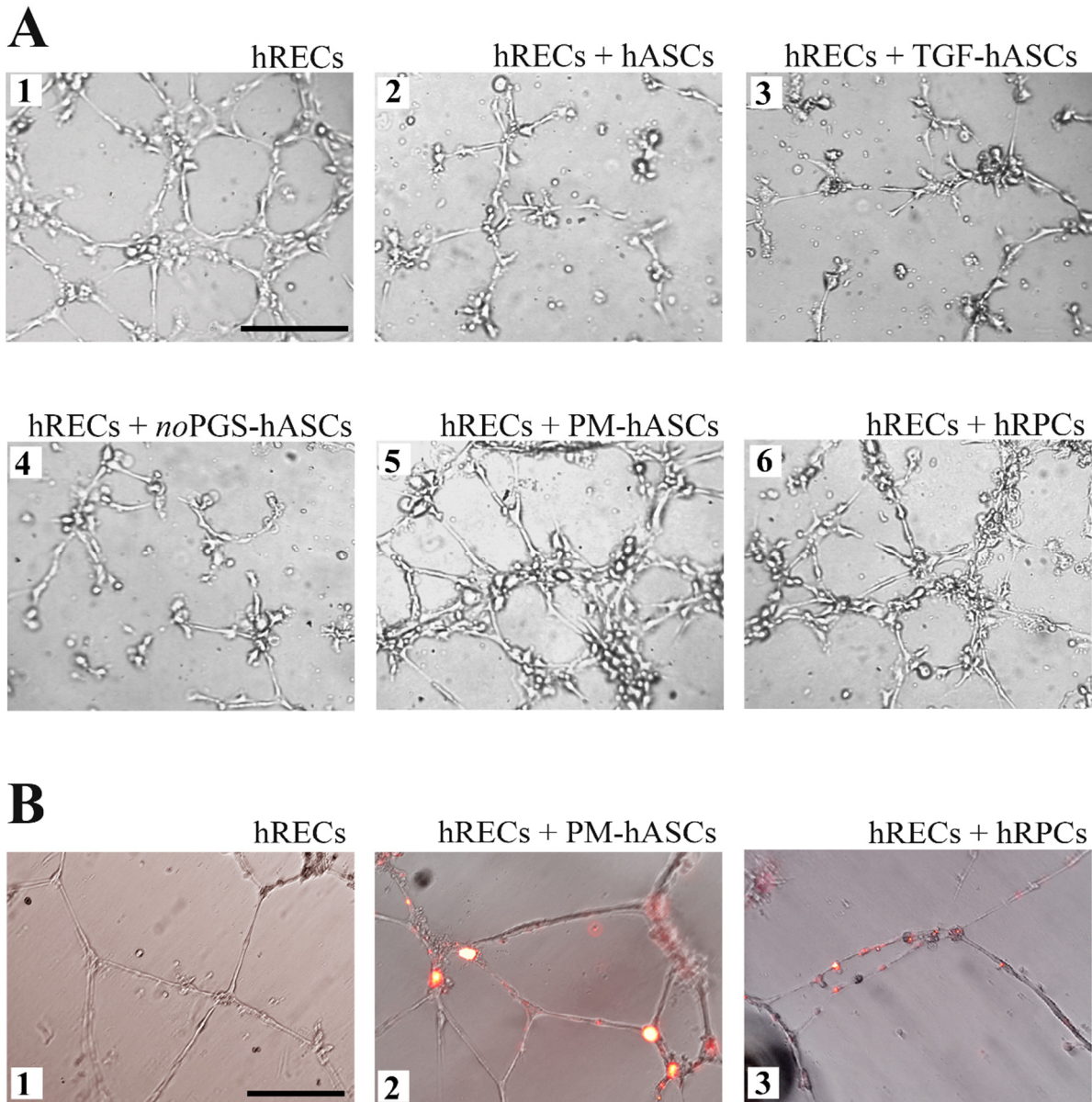PM-hASCs, hASCs pre-cultured in complete Pericyte Medium.

Figure 9 - *In vitro* three-dimensional cell cultures in Matrigel.

A) After 6 h from seeding, hRECs cultured on Matrigel spontaneously form tubular microvessel-like structures (1). This predisposition appears enhanced when hRECs are co-cultured with hRPCs (6) or PM-hASCs (5). No evident improvements are detectable if hRECs are co-cultured with control hASCs (2), TGF-hASCs (3) or *no*PGS-hASCs (4). Scale bar: 100 µm.

B) Typical tubular microvessel-like structures spontaneously formed by hRECs after 20 h from seeding (1). In 2 and 3, PM-hASCs and hRPCs, prelabeled with DiI (red fluorescence) were co-cultured with hRECs. In these cases, both cell types take the identical location in close proximity to the tubular formations. Scale bar: 50 µm.

Abbreviations:

hASCs, human Adipose derived mesenchymal Stem Cells pre-cultured in basal medium;

hRECs, human Retinal Endothelial Cells;

hRPCs, human Retinal Pericyte Cells;

TGF-hASCs, hASCs pre-stimulated with Transforming Growth Factor;

*no*PGS-hASCs, hASCs pre-cultured in Pericyte Medium lacking Pericyte Growth Supplement;

PM-hASCs, hASCs pre-cultured in complete Pericyte Medium.

8   Aim 2

Recent research shows that the intrastrial fluid-blood barrier might be a main point of entry for certain ototoxic drugs, including antibacterial aminoglycoside antibiotics such as gentamicin, anticancer agents such as cisplatin, and loop diuretics such as furosemide, from the blood into cochlear fluids and significantly induce hearing damage (Ding et al., 2012; Shi, 2016). In the physiology of the cochlea, PCs play a role in numerous cochlear pathologies, including, but not limited to, sudden sensorineural hearing loss, acoustic trauma, and inflammation of the cochlea. Understanding the role of PCs in pathophysiology is critical to develop novel protective strategies against drug-induced ototoxicity. The second research project has been carried out to obtain and cultivate primary BCPs, to be used for testing the effects of some ototoxic drugs. The use of BCPs for the analysis of ototoxic drugs can be a model to investigate pharmacological mechanisms and protection systems against different insults. The cochlea was isolated from just slaughtered bovine animals and immediately placed in culture. The isolated BCPs were characterized by immunostaining of pericyte markers: PDGFR, NG_2 and α-SMA. The ototoxic effects were evaluated to verify the susceptibility of the cochlear cells to some ototoxic drugs, cisplatin, gentamicin and furosemide; they were assessed by MTT viability test, cytotoxicity test by LDH (lactate dehydrogenase) production, detection of oxidative stress through the investigation of total

ROS by the 2',7'-dichlorofluorescin diacetate (DCDFDA) assay and MUSE cytofluorimetric analysis of the Annexin V to obtain the percentage of apoptotic cells. Dexamethasone, an anti-inflammatory drug, was added to assess its protective effect during treatment with the ototoxic drugs.

9   Chapter 2

Title: Isolation, cultivation and characterization of primary bovine cochlear pericytes: an new "*in vitro*" model of stria vascularis

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Running title: cultivation of primary bovine cochlear pericytes

Abstract

The study of strial pericytes is recently of great interest as they are pivotal for the physiology of stria vascularis, both in normal or pathological conditions. In order to limit the lack of *in vitro* model, here we described a growth medium-based approach to obtain and cultivate primary cochlear pericytes (BCP) from the stria vascularis of explanted bovine cochleae. We obtained high-quality pericytes in 8 – 10 days with a purity of >90% after the second passage. Immunocytochemical analysis showed a largely homogeneous population of cells that express the typical pericyte markers NG2, PDGFβ receptor, and alpha-smooth muscle actin α-SMA (expressed also by human retinal pericytes, HRPC) and were negative for endothelial marker vonWillebrand factor (expressed by human retinal endothelial cells, HREC). The sensitivity of BCPs to ototoxic drugs was evaluated through the treatment with cisplatin and gentamicin (tested at 2, 10, 50 and 250 µM) for 48 h and the analysis of cell viability (assessed by MTT). Compared to HREC and HRPC, cell viability of BCPs was significantly lower (p<0.05) after the treatment with 2 and 10 µM of gentamicin (about 20 and 15% respectively) and after the treatment with 50 and 250 µM of cisplatin (about 25 and 20% respectively). These data suggest that our simple and reliable method provides an effective method to culture highly pure strial pericytes from bovine and it could be is suitable for producing other primary culture cells from inner ear capillaries.

1. Introduction

The intrastrial fluid-blood barrier represents the homeostatic system of the inner ear. It tightly regulate the molecular trafficking from and to the bloodstream and maintain of the endolymph high potassium (K^+) levels required for the endocochlear potential (EP) (Hibino et al., 2010; Quraishi and Raphael, 2008; Salt et al., 1987). In the intrastrial fluid-blood barrier, endothelial cells (ECs) form a specialized capillary network characterized by a

unique polygonal organization, termed stria vascularis; perivascular cells, such as resident macrophage-like melanocytes (VM/Ms) and pericytes (PCs), participate to the structural complexity of the barrier (Shi, 2016; Juhn, 1988). A very large number of PCs surround the abluminal capillary wall of stria vascularis, embedded in the basal membrane (BM) and providing mechanical strength and physical elasticity to the vessels (Takeuchi et al., 2001). Strial PCs express neural/glial antigen 2 (NG2), CD90 (Thy-1), platelet-derived growth factor receptor-b (PDGFR□) intermediate filament protein and desmin (Shi et al., 2008; Neng et al. 2015).

PCs perform a broad range of functions such as vascular development and integrity, angiogenesis and tissue fibrogenesis (Allt and Lawrenson, 2001; Quaegebeur et al., 2010; von Tell et al., 2006). Strial PCs display a profound capacity to affect intrastrial fluid-blood barrier integrity through the regulation of tight junctions between ECs (Neng et al., 2012). PCs also contribute to BM formation by directly synthesizing type IV collagen, glycosaminoglycan, fibronectin, nidogen-1, perlecan, and laminin (Fisher, 2009; Shepro and Morel, 1993), and inhibit the activity of destabilizing matrix metalloproteinases (MMP), such as MMP-2 and MMP-9 (Zozulya et al., 2008). How strial PCs contribute to the composition and formation of strial BM is still unclear.

The impairment of the intrastrial fluid-blood barrier is closely related to the pathogenesis of a number of hearing disorders, such as autoimmune inner ear disease, Meniere's disease, meningitis-associated labyrinthitis and age-related ear loss (Zhang et al., 2015; Klein et al., 2008; Kamogashira et al. 2015). Moreover, the decrease of cochlear blood flow is a direct result of intrastrial fluid-blood barrier damage and is a common final pathophysiological hallmark associated to numerous inner ear diseases such as sudden sensorineural hearing loss and noise-induced hearing loss (Lamm and Arnold, 2000; Shi X., 2011). Studies showed that the capillary pericytes take an active role in the regulation of capillary blood flow in the cerebral cortex according to increased oxygen demand (Peppiatt et al., 2006,

Hall et al., 2014). Pericytes of the stria vascularis not only share the same embryological origin, but exhibit similar contractile phenotype, consistent with their pivotal role in the controlling of blood flow of the precapillary small arteries and arterioles like the spiral artery, (Dai et al., 2009, Scherer et al., 2010). Recently, it was been shown that the reduction of pre-capillaries diameter can be mediated by pericytes susceptibility to pro-inflammatory tumor necrosis factor (TNF) strongly contributing to the blood flow impairment in stria vascularis (Bertlich et al., 2017). Among the primary accessory cell in the intrastrial fluid-blood barrier, PCs showed profound morphological changes after LPS treatment in animal model, displayed a prominent round body and were found peeling away from the capillary wall, strongly contributing in barrier breakdown (Zhang et al. 2015).

Strong evidence indicate stria vascularis as target of damage induced by a variety of drugs, including antibacterial aminoglycoside antibiotics such as gentamicin and amikacin, anticancer agents such as cisplatin, carboplatin, nedaplatin, and oxaliplatin as well as loop diuretics such as furosemide (Palomar Garcia et al. 2001; Rybac et al. 2007).

Over the past few decades, in vitro cell-based models have been widely used for studying cell-cell interactions and regulation of blood barrier permeability in blood-brain-barrier (BBB) and blood-retina-barrier (BRB) (Duport et al., 1998; Lai and Kuo, 2005). Although different methods of isolation and culture of barrier cells are used to obtain barrier cells from different tissues (Bryan and D'Amore, 2008; Crisan et al., 2008; Mogensen et al., 2011), cell-based models are limited for BLB studies, due to the difficulty of isolating BLB component cells from the vestibular system. Cellular extraction methods generally used in non-cochlear tissues are set up for tissue where volume is not limited while are not suitable for small and anatomically complex systems such as cochlear microvasculature.

In this study, we describe a novel growth medium-based method associated to a mild enzymatic digestion to obtain primary PC from stria vascularis of bovine cochlea explants. Specific culture medium selectively supported the growth of PCs so that the unwanted cells

such as tissue-resident macrophages do not survived during the passages. This protocol was simple and allowed the production of primary PCs within 10-15 days with a purity of > 95% after two passages. Bovine has proved to be a beneficial source of cells for a lower ethical impact and the lack of housing costs. Our isolation method can increase the production of strial component cells and fill the gap in the understanding the functional role of pericytes in the stria vascularis.

2. Materials and Methods

2.1 Cochlear extraction

The cochlea is located in petrous bone, a spiral-shaped cavity in the bony labyrinth of the temporal bone (Fig 1d, e asterisk). To make the cochlea extraction the skull was reduced with a circular saw. First of all, a sagittal cut was made to divide the skull into two halves (Fig 1 b red dashed line); subsequently, after removal of the brain and dura mater, the hemi skull was reduced by cutting it transversely (Fig 1 b, c blue dashed line) and frontally (Fig 1 c green dashed line). The remaining part of the skull (Fig 1 d, scheme and Fig 1 e, photo) comprised part of the parietal bone (Fig 1 d, yellow), the temporal bone (Fig 1 d, blue) and the occipital bone (Fig 1 d, pink). A sagittal cut was made on the temporal bone (Fig 2 b) and after removal of a part of the bone the cochlea was highlighted in the bony labyrinth (Fig 2 c, d, e, f).

2.2 Reagents

Rabbit polyclonal antibody against NG2 (catalog num. ab129051) and rabbit polyclonal antibody against Von Willebrand Factor (vWF, catalog num. ab6994) were purchased by Abcam (Cambridge, UK). Mouse monoclonal antibody against alpha smooth muscle actin, (α-SMA, catalog num. M0851) was purchased by Dako (Agilent Technologies, Inc., Santa Clara, CA, USA). Mouse monoclonal antibody against platelet-derived growth factor receptor-β (PDGFR-β, catalog num. sc-374573) was purchased from Santa Cruz

Biotechnology, Inc. (Santa Cruz, CA). Dispase II protease from Bacillus polymyxa (catalog num. 04942078001) was from Roche GmbH (Roche Diagnostics, Indianapolis, IN). Gentamicin, cisplatin, Lipopolysaccharides from E.coli and human recombinant TNF-α were from Sigma-Aldrich (Milan, Italy).

2.3 Cell culture

Primary human retinal endothelial cells (HREC) and primary human retinal pericytes (HRPC) were purchased from Innoprot (Elexalde Derio, Spain). HREC were fed with culture EC medium, supplemented with 5% fetal bovine serum (FBS), 1% EC growth supplement, 100 U/ml penicillin and 100 µg/ml streptomycin. HRPC were fed with culture pericyte medium, supplemented with 5% fetal bovine serum (FBS), 1% pericyte growth supplement, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell media and supplements were purchased by Innoprot. Cells were plated in pre-treated T25 culture flasks (Costar; Corning, New York, US), with 0.01 mg/ml solution of poly-L-lysine (Innoprot, Elexalde Derio, Spain) in sterile-filtered water for 1h at 37°C and then rinsed twice with sterile water. Cells were incubated at 37 °C with 5 % CO_2 until reaching about 70% confluence.

2.4 Isolation of primary bovine cochlear pericytes (BCP)

All procedures were performed under aseptic conditions. The cochleae were isolated by a bovine directly after killing in the slaughterhouse, were immediately placed into ice-cold phosphate-buffered saline (PBS) containing 100 U/mL penicillin and 100 µg/mL streptomycin. Collection of two cochleae yielded enough cell for the propagation of the cell line. The transport of the cochleae in laboratory was made in less than 1h. Before starting the pericyte isolation, a sterile work area was set up in a laminar flow hood and were placed a sets of scissors and forceps, scalpel, PBS 1X (ice-cold), enzyme solution, wash medium, and selection culture medium in the tissue culture hood. All cochleae were washed twice in

ice-cold 1X PBS to prevent contamination before use in further steps. Each cochlea was placed separately in 60-mm-diameter tissue culture dish (Costar; Corning, New York, US), with ice-cold 1X PBS and they were finely cut using sterile scissors, scalpel and forceps. For each cochlea, all the fragmented pieces were incubated in a 50 ml tubes with 1 ml of fresh prepared digestion solution (dispase II, 0.8U/mL in PBS 1X) for 1h at 37°C under slow agitation. An additional mechanical disruption was performed by carefully pipetting the solution during the incubation, facilitating the production of cell suspension from stria vascularis. After the incubation period, digestion was stopped by adding EDTA (final conc. 1mM) and the solution was repeatedly pipetted up and down contributing to dissolve the cellular aggregates. Then, cell suspensions were filtered using a 100 □m cell strainer of nylon (Falcon, New York, US) to separate the dispersed cells and small tissue fragments from the larger piece. The filtrates were collected in 15 ml tubes and were centrifuged at 400 g for 5 min at +4°C. The supernatant was discarded and the precipitated pellet containing cells and capillary-rich small fragment was re-suspended in 10.5 ml of specific growth medium, containing pericyte medium, supplemented with 5% fetal bovine serum (FBS), BSA 10 µg/ml, apo-transferrin 10 µg/ml, insulin 5 mg/ml, EGF-2 2 ng/ml, FGF-2 2ng/ml, IGF-I 2 ng/ml and hydrocortisone 1 mg/ml, 100 U/ml penicillin and 100 µg/ml streptomycin (Innoprot, Elexalde Derio, Spain). Cells were seeded in 2 T25 flask (Costar; Corning, New York, US), pre-treated with 0.01 mg/ml solution of poly-L-lysine (Innoprot, Elexalde Derio, Spain) in sterile-filtered water for 1h at 37°C and then rinsed twice with sterile water.and incubated at 37°C with 5% CO2 atmosphere. The isolation procedure was carried out as fast as possible in order to minimize cell damage.

2.5 Cultivation of BCPs and morphological characterization

The medium was changed for the first time 4 days after the cell seeding and then every 3 days. When primary BCPs cultures were near confluence (above 80%), they were further

propagated by treatment with 1 mL of 0.25% trypsin and 0.02% EDTA (Life Technologies, Monza, Italy) for 3 minutes and then were either split into a 1:3 ratio to passage to additional 6-well plates or harvested for cryopreservation. Cell growth and phenotype were monitored using bright field microscopy equipped with CCD camera Optica. Bright field microscopy was used to carried out comparative analysis of cell shape in HREC, BCP and HRPC. 15×10^3 cells (passage 3) were seeded in coated 60 mm Petri dishes and incubated at 37°C with 5% CO2 atmosphere for 24h. Then, medium was replaced with fresh medium without or with 10 ng/ml of TNF-α or 100 LPS. After 48h representatives images were captured with bright field microscopy equipped with CCD camera Optica and images were converted in grey scale.

2.6 Immunofluorescence analysis

Immunocytochemistry was carried out to identify in cultured cells the presence of typical pericyte surface markers in comparison to known cell line as HREC and HRPC. For immunocytochemistry, cells at passage 3 were sub-cultured on glass coverslips, placed in a 24-well plate. Coverslips were previously treated with a 0.01 mg/ml poly-L-lysine solution (PLL, Innoprot) in sterile-filtered water for 1 h at 37°C and then washed twice with sterile water. Cells were seeded on PLL-coated glass coverslips at densities of 20000 cells/well and incubated for 2 days at 37°C in a humidified atmosphere of 5% CO_2. Then, cells were washed with PBS 1X, fixed with 4 % paraformaldehyde in PBS 1X for 30 min and incubated for 30 min in a 5% solution of normal goat serum and 0.1% triton in PBS 1X. Finally, cells were incubated overnight at 4 °C with primary antibodies in a PBS 1X/triton 0.1% solution. Antibody concentrations were: anti-NG2; 1:90, anti-PDGFRβ; 1:90, anti-αSM; 1:120 and anti-vWF; 1:120. The following day, cells were washed with PBS 1X and incubated for 1 h at room temperature with FITC-conjugated goat anti-rabbit secondary antibody or with Cy3-

conjugated goat anti-mouse (Abcam) in the dark. In order to assess the specificity of immunostaining we carried out control samples by omitting the primary or secondary antibodies during the incubations for each sample analyzed. Cell nuclei were stained with DAPI (Life Technologies, Monza, Italy) for 10 min 1:10000), at room temperature in the dark. Slides were then mounted using mounting medium (Life Technologies) and observed using a Leica inverted fluorescence microscope equipped with a computer assisted Nikon digital camera. (Carl Zeiss, Oberkochen, Germany).

2.7 Cell viability

Cellular viability was assessed using the 3-[4,5-dimethylthiazol-2-y l]-2,5-diphenyl tetrasodium bromide (MTT assay, Chemicon, Temecula, CA) as described elsewhere (Giurdanella et al, 2017). Optimal cell density was obtained seeding 15000 cells/well in 96-well plates. After 24h of culture, the medium was replaced with fresh medium without drugs or supplemented with different concentration of cisplatin or gentamicin ranging from 2 to 250 μM. At the end of the treatment (48h), cells were first incubated at 37°C with MTT (0.5 mg/ml) for 4 h; then 100μl isopropanol/0.04 N HCl (1:10) was added and, after 1 h, absorbance was measured at 570nm in a plate reader (VariosKan, Thermo Fisher Scientific, Waltham, MA).

2.8 Statistical analysis

Statistical significance between two groups was analyzed by Student's t-test. Two-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test, was used for multiple comparisons. P values< 0.05 were considered statistically significant.

3. Results

3.1 Cultivation of BCP

We first attempt to obtain primary pericytes from bovine cochleae by plating the cells achieved after the isolation protocol (described in materials and methods section) in the specific growth medium. Heterogeneous cells, tissues fragments and residual red blood cells were observed immediately after plating cultures. The cells started to be adherent to culture dishes within 12-24 hours. Culture medium was changed for the first time after 72h from the plating of the cells, when unattached contaminating cells were discarded. Remained BCPs showed irregular triangular morphology and very few cells appeared to have smaller and rounder cell bodies compatible with monocytes. We did not observe any close-patterns typical of ECs cultures or any characteristic perivascular resident macrophage-type melanocytes [Neng et al. 2012]. Contaminating cells were gradually eliminated by rinsing plates when medium was changed on subsequent days. This resulted in only very few contaminating cells left in culture (Fig. 1). On day 5, BCPs appeared as either isolated cells containing typical stress fibers or as loose colonies of few cells with branched shape with a polygonal cell body, large nuclei and long processes (Shi et al., 2008) (Fig. 1, panels a and a'). On day 9, both single and scattered cells began to form cell colony or previous little cell colonies sparsely spread on the bottom of the flask (Fig. 1, panels b and b'). Growth of the primary BCPs continued throughout the next days maintaining the same phenotype as showed in representative images of Fig.1, panels c, c', d and d', acquired respectively at days 14 and 18 where cells reach approximated 80% confluence. Total confluence was achieved at day 23 (Fig. 1, panels f and f'. The subcultures grew faster than the primary cultures and reached confluence on days 12-14. BCPs were successfully cryopreserved and recultured without loss of typical phenotype. Cells were first passed at 1:2 ratio and then were repeatedly passed until 9 times at 1:3 ratio without loss of characteristic phenotype throughout the passages. BCPs were primarily analyzed and characterized based upon morphology under phase contrast microscopy. Cells were confirmed as BCPs by observing a typical pericyte morphology with irregular shape, long

processes, and large and flat cell bodies [9, 38-41]. In the cultures, almost all of the cells showed typical pericyte morphology.

3.2 Characterization of pericytes markers in BCPs

Based on the previous observations, we further analyzed BCPs phenotype assessing their positivity for the typical pericytes markers α-SMA, PDGFR-β, NG2 and the also the negativity for the endothelial marker von Willebrand factor (Shi et al., 2008, Franz et al., 2004) in comparison with the well characterized cell line HRPC and HREC (control cell lines). Immunocytochemical analysis showed a specific staining for NG2, PDGFR-β and α-SMA in 99% of BCPs (Fig 3. panels A', B' and C', respectively), in 99% of positive control cell line HRPC (Fig. 3. Panels A'', B'' and C'' respectively), and a completely negative signal for von Willebrand factor (Fig 3. panels D' and D''). These data confirmed both the high purity of BCPs in culture and the same pattern of molecular markers of pericytes. Moreover, as shown in Fig. 3 panel C', α-SMA immunofluorescent signal showed a typical organized pattern of a cytoskeletal protein. As expected, HREC (negative control cell line) were did not show fluorescent staining for NG2, PDGFR-β and α-SMA (Fig. 3. panels A, B and C respectively) while were positive for von Willebrand factor (Fig. 3. panel D) confirming the previous immunocytochemical analysis. The expression of these 4 markers did not significantly change in BCPs from passages 3 to 7. There was no contamination of cultures by ECs. The negative staining controls that was carried out either with only primary antibodies or with only secondary antibodies all showed no signals.

3.3 Pericytes shape changes induced by pro-inflammatory mediators

Several studies have shown the ability of pericytes to exhibit morphology changes after stimulation with pro-inflammatory cytokines in vitro, such as TNF-α. (Proebstl et al. In order

to test this phenomenon in our model, BCP were treated with submaximal concentration of TNF-α (10 ng/ml) or lipopolysaccharide (LPS, 100 ng /ml) for 48h. Bright field microscopy images were acquired at the end of treatment and were compared to images from HREC and HRPC cultured in the same conditions. Results showed a profound changing in cell shape of BCP in response to both TNF-α and LPS producing an increase in cell length, a longer processes and a more branched shape in comparison to untreated cells (Fig. 5 panels A', B' and C'). The same treatments induced a relevant effect on HRPC shape increasing cell processes length and the cell body size referred to control (Fig. 5 panels A'', B'' and C''). Bright field microscopy highlighted very mild changes in shape of treated HREC indicating a strong responsiveness of pericytes to TNF-α and LPS.

3.4 Susceptibility of BCPs to ototoxic drugs

To mimic in vitro the insult to stria vascularis induced by ototoxic drugs, BCPs were treated with cisplatin and gentamicin. Treatments were carried out at different concentrations ranging both from 2 to 250 µM, for 48h and cell toxicity was evaluated through the evaluation of mitochondrial function (MTT assay). The same treatment was performed also on HRPC and HREC and the data were compared to BCP treatmens. As shown in Fig.3, MTT was reduced by

4. Discussion

Over the past decades, in vitro cell-based models spread widely as powerful tools enabling us to understand the molecular and cellular processes in different cell types, both in normal or in pathological conditions. Such *in vitro* models can reach a very high degree of elegance,

combining different cell line in co-culture or triple-culture systems (Neng et al. 2013). The development of increasingly relevant models is based on adequate cellular extraction methods in order to produce primary cell lines from different starting tissue sources. Most of the methods are time-consuming and involve multiple steps of enzymatic digestion, gradient density centrifuging and glass bead or magnetic, fluorescence activated cell sorting (FACS), magnetic beads, or antibody-based approaches to obtain pure cultures. Despite these approaches are effective in increasing the purity of cell cultures, they tend to reduce cell yield, require high number of passage and could cause some damage to the cell (Bernas et al., 2010). Culture media-based selection and growth of primary cell is based on a specific culture media formulated to selectively growth certain cell phenotypes starting from a specific cell source. Such methods showed to be very reliable providing consistent results. (Zhang et al., 2017). To our knowledge, we first describe a protocol for culturing strial PCs from bovine cochlea. Bovine cell cultures are extremely used and provided extensive evidence about their validity as in vitro models for studies of molecular pathways involved in many pathological conditions, (Lupo et al., 2013, Primo and Arboleda-Velasquez, 2016) Moreover, cochleae are extracted from animals killed in the slaughterhouses providing numerous advantages given by the lack of animal housing. Moreover, the use of such animals imposes certainly less ethical issues, giving them more usefulness and dignity. Our cell extraction method differs from previously published because it combine a mild enzymatic digestion with collagenase and a very fast sieving step for separating large tissue fragments of digested cochleae from single cell. These steps are necessary to recover cells from a complex organ as the cochlea limiting as much as possible cell damage induced by extraction procedure. Moreover, strial PCs are selected by using a specialized media. PCs proliferation is promoted by adding to the medium a specific combination of growth factors such as insulin, EGF-2, FGF-2, IGF-I, with BSA, apo-transferrin and hydrocortisone. This culture condition is similar to what we used for other PC cell line (HRPC) as described in our

previous work (Giurdanella et al., 2015). Pericytes are morphologically, biochemically, and physiologically heterogeneous cells with a contractile phenotype (Sims et al., 2000). In this study, strial PCs were validated through both the expression profiling of specific cell markers and the ascertaining of morphological characteristics. Here we tested the expression of PDGFR□, □SMA and NG2 as used to characterize other PCs (Armulik et al. 2005, Shi et al. 2008). Generated PCs cell line purity was determine by the evaluation of specific protein marker for endothelial cells vonWillebrand factor in immunocytochemical analyses.

Despite bovine source of pericytes impose an anatomical approach to recover the cochleae, the yield of primary PCs generated was higher than from mouse cochleae. For example, approximately 5×10^6 PCs at P3 were obtained from stria vascularis of two bovine cochleae compared to $1\text{-}2 \times 10^6$ PCs generated from six cochleae of mouse (Neng et al. 2013). The production of this cell-based in vitro model allows us to investigate the role of the PCs in the modulation of the intrastrial fluid-blood barrier stability and diameter in inflammatory and ototoxic conditions. Many studies show that some ototoxic drugs cause structural damage of stria vascularis, producing an enhanced drug uptake from the blood into cochlear fluids and an increased hearing damage (Campbell et al. 1999). Moreover, Breglio and co-workers recently reported a long-term high accumulation of cisplatin into the stria vascularis indicating it as an important therapeutic target for preventing cisplatin ototoxicity (Breglio et al. 2017). All these findings suggest that direct studies of the strial blood barrier functions can represent a useful approach in order to test reliable strategy for preventing the dysfunction of the stria vascularis. As reported in our study, the treatment with ototoxic drugs showed a higher sensitivity of bovine strial PCs, indicating this cell line as cell-based in vitro model to study drug ototoxicity or inflammatory stimuli.
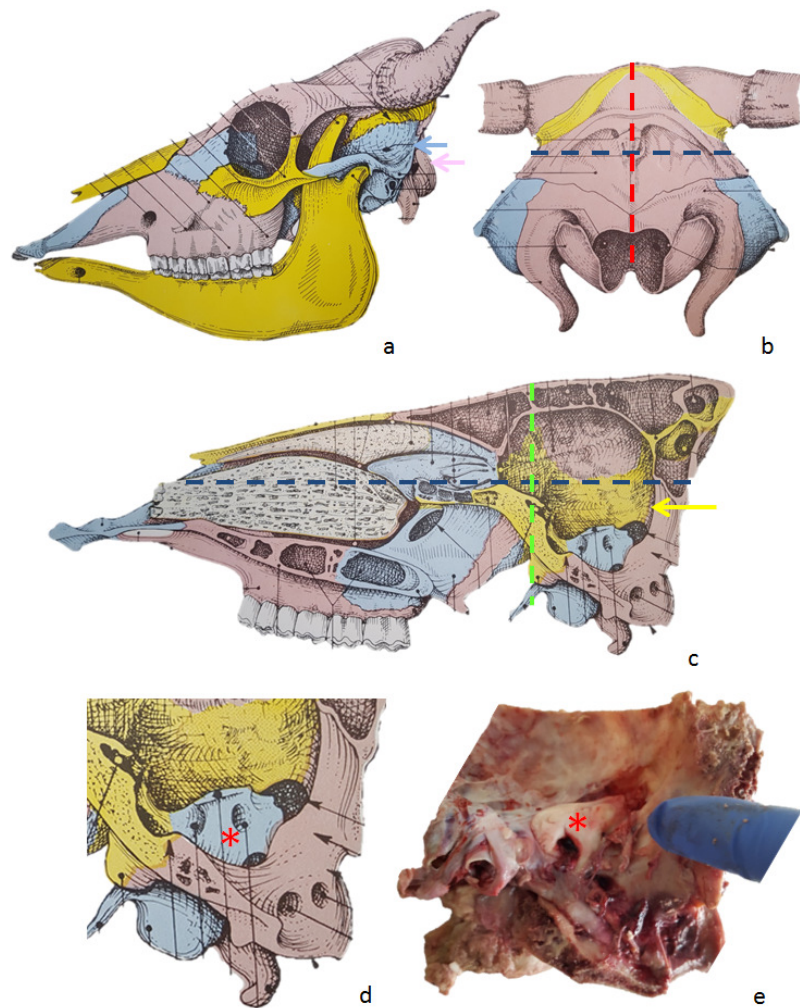
FIGURES

Fig 1. – Scheme of bovine skull modified (From Barone – Atlante di osteo-artro-miologia del cavallo e del bovino); a) lateral view of skull; temporal bone where the inner ear is located (blue arrow); occipital bone (pink arrow). b) nuchal face of bovine skull; red and blue dashed lines represent the cut made to isolate the inner ear; the only remaining part of the parietal bone (yellow), the temporal bone (blue) and the occipital bone (pink). c) sagittal section of bovine skull; blue and green dashed lines represent the cut to reduce the skull; d) magnification of the remaining portion of the skull after making reduction cuts comprising part of the parietal bone (yellow), the temporal bone (blue) represented by petrous bone

(asterisk) and the occipital bone (pink). e) photo of skull portion used for cochlea extraction located in petrous portion of temporal bone (asterisk).
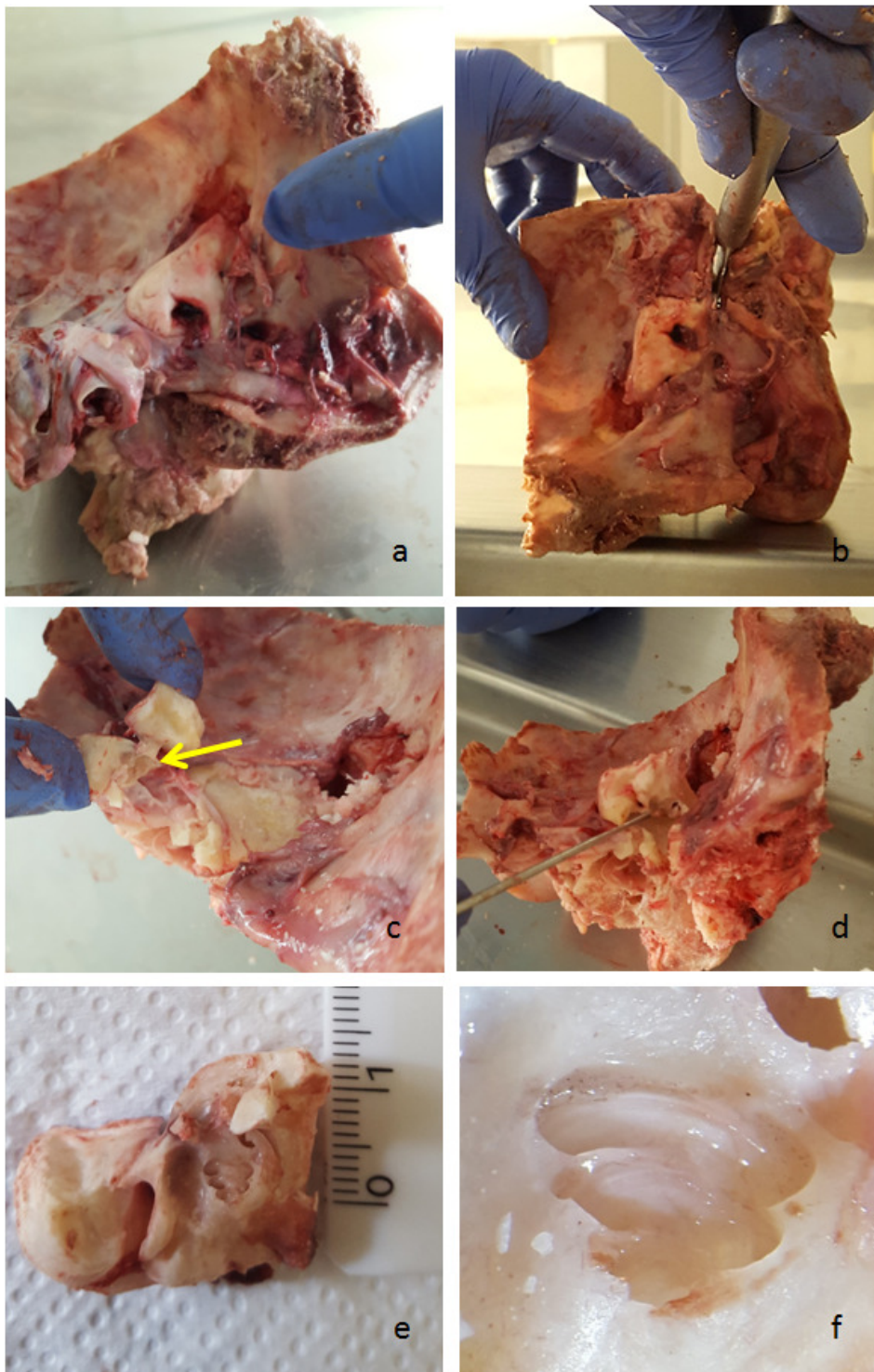

Fig 2. – Sequence of cochlea extraction; a) Internal view of occipital and temporal bones after brain and dura mater removal. b) Sagittal section of temporal bone starting at external

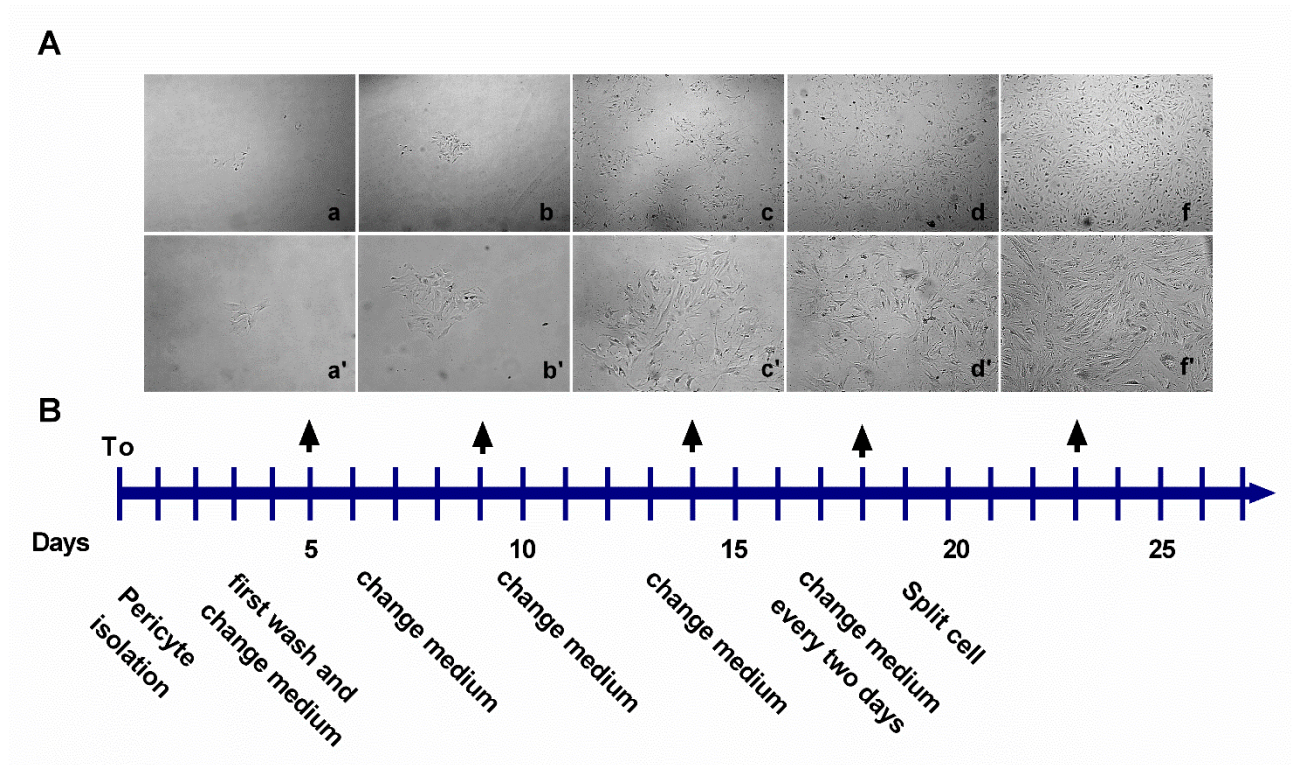acoustic meatus. c,d) Opening of petrous bone and highlight of cochlea (yellow arrow). e,f) magnification of petrous bone containing the cochlea.

Fig 3. General timing of BCP isolation and cell phenotype, showing flat and irregular-shaped cells with many processes. Representative bright field microscopy images of BCP at different stages of growth and acquired with 10x (a – f) or 20x (a' – f') objective. Little clones of dispersed cell were visible at 5 days from stria vascularis fragments plating that achieve cell confluence in about 20 days. Scale bar: 100 μm
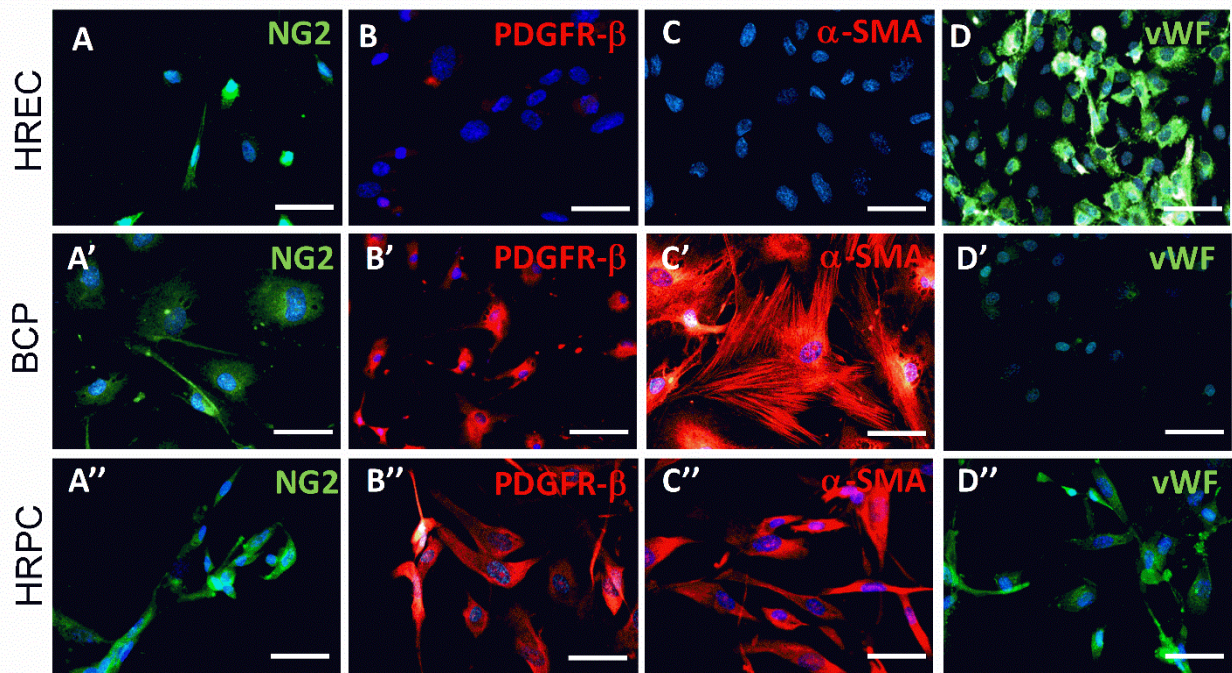
Fig 4. Characterization of isolated BCP at passage 3 by immunostaining of pericyte molecular markers. Green fluorescence indicate the immunostaining for NG2 (panels A, A' and A'') and vWF (panels D, D' and D'') in HREC, BCP and HRPC respectively. Red fluorescence indicates immunostaining for PDGFRβ (panels B, B' and B'') and α-SMA (panel C, C' and C'') in HREC, BCP and HRPC respectively. Blue fluorescence depicts cell nuclei, stained by DAPI. Cells were at passage 3 in ECM 5% FBS (HREC) and PC Medium 5% FBS (BCP or HRPC). No immunostaining is visible for markers considered typical for pericytes as PDGFRβ or αSMA in HREC (panels B and C). The presence of immunonegative cells is documented by DAPI nuclear fluorescence. Virtually every cell of BCP or HRPC samples are immunopositive for pericyte markers (panels A',B', C', A'', B'' and C''). Magnification: 20x; scale bars: 100 μm.
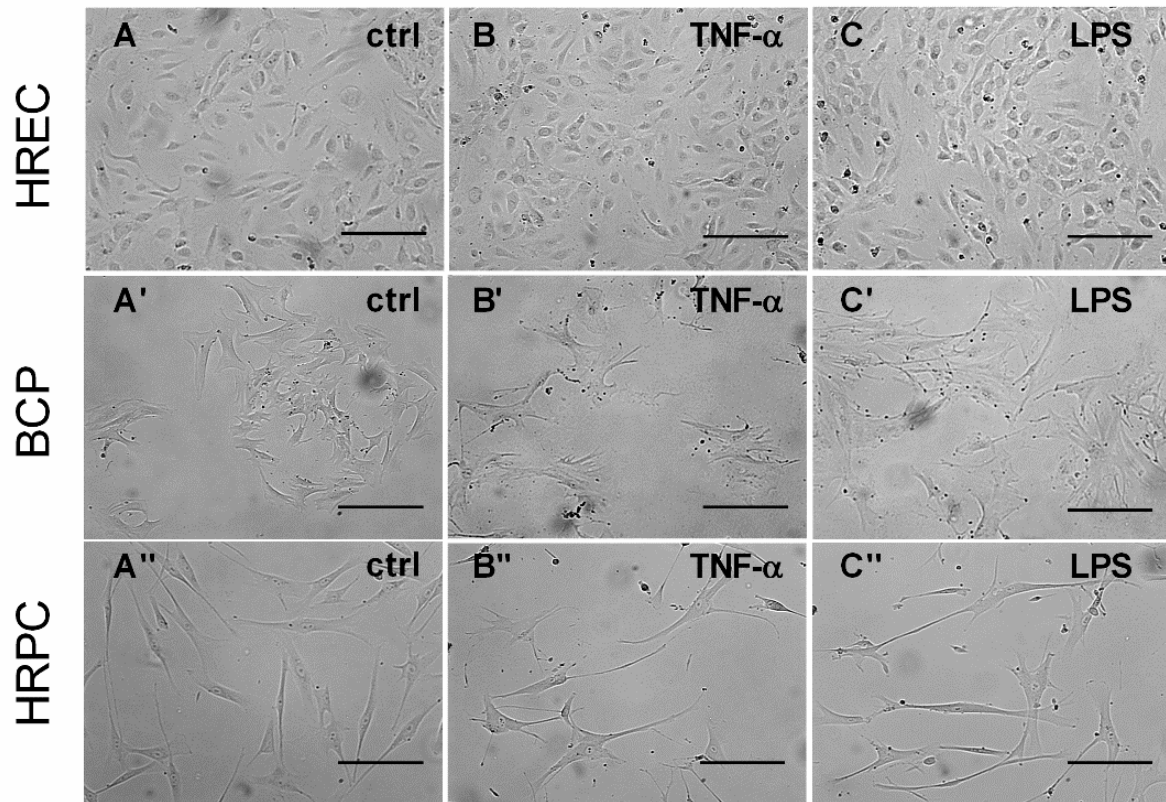
Fig. 5. Effects of TNF-α and LPS treatment on cell shape of HREC, BCP and HRPC in vitro. Representative bright field microscopy images are shown of unstimulated cells (ctrl, panels A, A' and A'') or after 48h of treatment with TNF-α (10ng/ml, panels B, B' and B'') or with LPS (10ng/ml, panels C, C' and C'') in HREC, BCP and HRPC respectively. Cells were at passage 3. Magnification: 20x; scale bars: 100 µm.
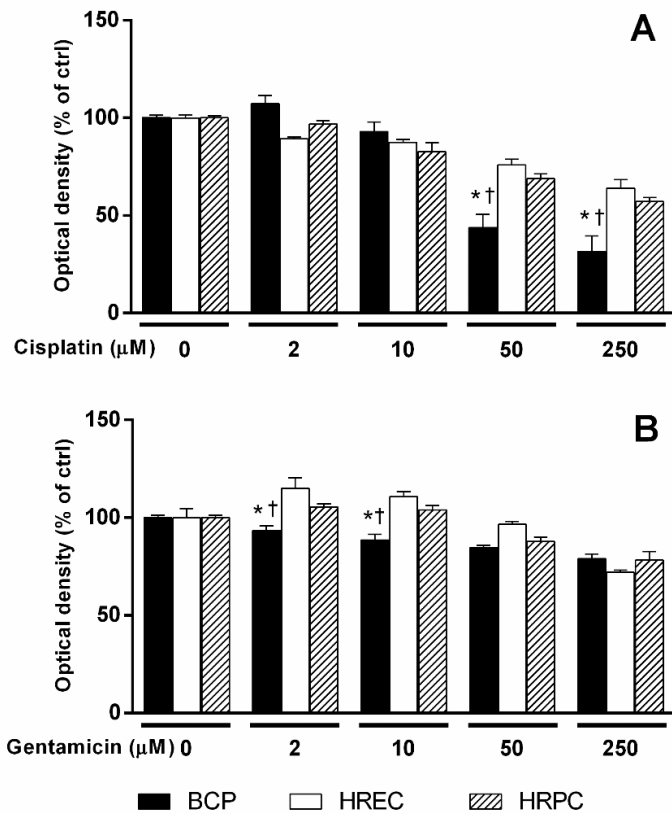
Fig 6. Effect of cisplatin and gentamicin on cell viability of BCPs, HRECs and HRPCs. Cells (passage 3) were cultured for 48h in the medium without drugs (control condition, 0 □M) or with different concentrations of cisplatin (panel A) and gentamicin (panel B). Cell viability values are reported as a mean ± SEM of three independent experiments, each involving six different wells per condition. *P < 0.05 vs. HREC in the same condition of treatment, †P < 0.05 vs. HRPC in the same condition of treatment, Two-way ANOVA, followed by Bonferroni's comparison test.

Reference

- Allt, G., Lawrenson, J., 2001. Pericytes: cell biology and pathology. Cells Tissues Organs 169, 1-11.
- Armulik, A., Abramsson, A., Betsholtz, C., 2005. Endothelial/pericyte interactions. Circ. Res. 97, 512-523.
- Bernas, M.J., Cardoso, F.L., Daley, S.K., Weinand, M.E., Campos, A.R., Ferreira, A.J., Hoying, J.B., Witte, M.H., Brites, D., Persidsky, Y., Ramirez, S.H., Brito, M.A., 2010.Establishment of primary cultures of human brain microvascular endothelial cells to provide an in vitro cellular model of the blood-brain barrier. Nat. Protoc. 5, 1265-1272.
- Bertlich, M., Ihler, F., Weiss, B.G., Freytag, S., Strupp, M., Canis, M., 2017. Cochlear pericytes are capable of reversably decreasing capillary diameter in vivo after tumor necrosis factor exposure, Otol. Neurotol. 38 (10), 545-550.
- Breglio A.M., Rusheen A.E., Shide E.D., Fernandez K.A., Spielbauer K.K., McLachlin K.M., Hall M.D., Amable L., Cunningham L.L., 2017. Cisplatin is retained in the cochlea indefinitely following chemotherapy. Nat Commun. Nov 21; 8:1654, 1-9.
- Bryan, B.A. and D'Amore P.A., 2008. Pericyte isolation and use in endothelial/pericyte coculture models. Methods Enzymol; 443: 315-31.
- Campbell, K.C., Meech, R.P., Rybak, L.P., Hughes, L.F., 1999. D-Methionine protects against cisplatin damage to the stria vascularis. Hear Res. 138, 13-28.
- Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., Péault, B., 2008. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. Methods Cell Biol., 86: 295-309.
- Dai, M., Nuttall, A., Yang, Y., Shi, X., 2009. Visualization and contractile activity of cochlear pericytes in the capillaries of the spiral ligament. Hear. Res. 254: 100–107.
- Duport, S., Robert, F., Muller, D., Grau, G., Parisi, L., Stoppini, L., 1998. An in vitro blood-brain barrier model: cocultures between endothelial cells and organotypic brain slice cultures. Proc. Natl. Acad. Sci. U. S. A. 95, 1840-1845.
- Fisher, M., 2009. Pericyte signaling in the neurovascular unit. Stroke 40, S13-S15.
- Franz, P., Helmreich, M., Stach, M., Franz-Italon, C., Böck, P., 2004. Distribution of actin and myosin in the cochlear microvascular bed. Acta Otolaryngol. 124, 481-485.
- Giurdanella, G., Anfuso, C.D., Olivieri, M., Lupo, G., Caporarello, N., Eandi, C.M., Drago, F., Bucolo, C., Salomone, S., 2015. Aflibercept, bevacizumab and ranibizumab prevent glucose-induced damage in human retinal pericytes in vitro, through a PLA2/COX-2/VEGF-A pathway. Biochem Pharmacol. 2015 Aug 1; 96(3):278-87.
- Giurdanella, G., Lazzara, F., Caporarello, N., Lupo, G., Anfuso, C.D., Eandi, C.M., Leggio G.M., Drago, F., Bucolo, C., Salomone, S., 2017. Sulodexide prevents activation of the PLA2/COX-2/VEGF inflammatory pathway in human retinal endothelial cells by blocking the effect of AGE/RAGE. Biochem Pharmacol. 2017 Oct 15;142:145-154.
- Hall, C.N., Reynell, C., Gesslein, B., Hamilton, N.B., Mishra, A., Sutherland, B.A., O/ 'Farrell, F.M., Buchan, A.M., Lauritzen, M., Attwell, D., 2014. Capillary pericytes regulate cerebral blood flow in health and disease. Nature 508, 55-60.

- Hibino, H., Nin, F., Tsuzuki, C., Kurachi, Y., 2010. How is the highly positive endocochlear potential formed? The specific architecture of the stria vascularis and the roles of the ion-transport apparatus. Pflugers Arch. 459: 521-533.
- Juhn, S.K., Rybak, L.P., 1981. Labyrinthine barriers and cochlear homeostasis. Acta Otolaryngol. 91, 529-534.
- Kamogashira, T., Fujimoto, C., Yamasoba, T., 2015. Reactive oxygen species, apoptosis, and mitochondrial D\dysfunction in hearing loss. Biomed. Res. Int. 2015.
- Klein, M., Koedel, U., Kastenbauer, S., Pfister HW, 2008. Nitrogen and oxygen molecules in meningitis-associated labyrinthitis and hearing impairment. Infection 36: 2–14.
- Lai, C.-H., Kuo, K.-H., 2005. The critical component to establish in vitro BBB model: Pericyte. Brain Res. Rev. 50: 258-265.
- Lamm, K., Arnold, W., 2000. The effect of blood flow promoting drugs on cochlear blood flow, perilymphatic pO(2) and auditory function in guinea pig inner ear. Hear Res;141:199-219
- Lupo G, Motta C, Giurdanella G, Anfuso CD, Alberghina M, Drago F, Salomone S, Bucolo C, 2013. Role of phospholipase A2 in diabetic retinopathy: in vitro and in vivo studies. Biochem Pharmacol. 2013 Dec 1;86(11):1603-13.
- Juhn, S.K., 1988. Barrier systems in the inner ear. Acta Otolaryngol. Suppl. 458, 79-83.
- Mogensen, C., Bergner, B., Wallner, S., Ritter, A., d'Avis, S., Ninichuk, V., Kameritsch, P., Gloe, T., Nagel, W., Pohl, U., 2011. Isolation and functional characterization of pericytes derived from hamster skeletal muscle. Acta Physiol (Oxf). Apr; 201(4): 413-26.
- Neng, L., Zhang, F., Kachelmeier, A., Shi, X., 2012. Endothelial cell, pericyte, and perivascular resident macrophage-type melanocyte interactions regulate cochlear intrastrial fluid-blood barrier permeability. J. Assoc. Res. Otolaryngol. 14, 175-185.
- Neng, L., Zhang, W., Hassan, A., Zemla, M., Kachelmeier, A., Fridberger, A., Auer, M., Shi, X., 2013. Isolation and culture of endothelial cells, pericytes and perivascular resident macrophage-like melanocytes from the young mouse ear. Nat. Protoc. 8, 709-720.
- Neng, L., Zhang, J., Yang, J., Zhang, F., Lopez, I.A., Dong, M., Shi, X., 2015. Structural changes in the strial blood-labyrinth barrier of aged C57BL/6 mice. Cell Tissue Res. 1-12.
- Palomar García, V., Abdulghani Martínez, F., Bodet Agustí, E., Andreu Mencía, L., Palomar Asenjo, V., 2001. Drug-induced otoxicity: current status. Acta Otolaryngol
- Peppiatt, C.M., Howarth, C., Mobbs, P., Attwell, D., 2006. Bidirectional control of CNS capillary diameter by pericytes. Nature 443, 700-704.
- Primo VA and Arboleda-Velasquez JF., 2016. Isolation and Transfection of Primary Culture Bovine Retinal Pericytes. Methods Mol Biol. 2016;1430: 107-17.
- Proebstl D., Voisin M.B., Woodfin A., Whiteford J., D'Acquisto F., Jones G.E., Rowe D., Nourshargh S. (2012). Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. J Exp Med. Jun 4;209(6): 1219-34
- Quaegebeur, A., Segura, I., Carmeliet, P., 2010. Pericytes: blood-brain barrier safeguards against neurodegeneration? Neuron 68, 321-323.
- Quraishi, I.H., Raphael, R.M., 2008. Generation of the endocochlear potential: a biophysical model. Biophys. J. 94, L64-L66.
- Rybak, L.P., Whitworth, C.A., Mukherjea, D., Ramkumar, V., 2007. Mechanisms of cisplatin-induced ototoxicity and prevention. Hear Res. 226, 157-167.
- Salt, A.N., Mleichar, I., Thalmann, R., 1987. Mechanisms of endocochlear potential generation by stria vascularis. Laryngoscope 97: 984-991.

- Scherer, E.Q., Yang, J., Canis, M., Reimann, K., Ivanov, K., Diehl, C.D., Backx, P.H., Wier, W.G., Strieth, S., Wangemann, P., Voigtlaender-Bolz, J., Lidington, D., Bolz, S.S., 2010. Tumor necrosis factor-alpha enhances microvascular tone and reduces blood flow in the cochlea via enhanced sphingosine-1-phosphate signaling. Stroke 41, 2618–2624.
- Shepro, D., Morel, N., 1993. Pericyte physiology. FASEB J. 7, 1031-1038.
- Shi, X., Han, W., Yamamoto H., Tang, W., Lin, X., Xiu, R., Trune, D. R., and Nuttall, A. L., 2008. The Cochlear Pericytes. Microcirculation, Aug: 15(6), 515-529.
- Shi, X., 2011. Physiopathology of the cochlear microcirculation, 2011. Hear Res 282: 10-24.
- Shi, X., 2016. Pathophysiology of the cochlear intrastrial fluid-blood barrier. Hear Res., Aug, 338: 52-63.
- Sims, D.E., 2000. Diversity within pericytes. Clin Ex-per Pharmacol Physiol. 27: 842–846.
- Takeuchi, S., Ando, M., Sato, T., Kakigi, A., 2001. Three-dimensional and ultrastructural relationships between intermediate cells and capillaries in the gerbil stria vascularis. Hear Res. 155, 103-112.
- von Tell, D., Armulik, A., Betsholtz, C., 2006. Pericytes and vascular stability. Exp. Cell Res. 312, 623-629.
- Yang, Y., Dai, M., Wilson, T.M., Omelchenko, I., Klimek, J.E., Wilmarth, P.A., David, L.L., Nuttall, A.L., Gillespie, P.G., Shi, X., 2011. Naþ/Kþ-ATPase a1 identified as an abundant protein in the blood-labyrinth barrier that plays an essential role in the barrier integrity. PLoS One.
- Zhang, J., Chen, S., Hou, Z., Cai, J., Dong, M., Shi, X., 2015. Lipopolysaccharideinduced middle ear inflammation disrupts the cochlear intra-strial fluid-blood barrier through down-regulation of tight junction proteins. PLoS One 10(3).
- Zhang, J., Chen, S., Cai, J., Hou, Z., Wang, X., Kachelmeier, A., Shi, X., 2017. Culture media-based selection of endothelial cells, pericytes, and perivascular-resident macrophage-like melanocytes from the young mouse vestibular system. Hear Res. 345, 10-22.
- Zozulya, A., Weidenfeller, C., Galla, H.-J., 2008. Pericyte-endothelial cell interaction increases MMP-9 secretion at the bloodebrain barrier in vitro. Brain Res. 1189, 1-11.

10 Chapter 3

Effects of ototoxic drugs on a new in vitro model of stria vascularis*

* Poster presented at 39° National Congress of the Italian Society of Pharmacology, Firenze 20-23 november 2019

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Background

Strial pericytes are an essential component of stria vascularis (Blood Labyrinth Barrier) for maintaining and regulating homeostatic conditions in the inner ear. Strial pericytes were shown impaired in different pathological conditions and/or by a variety of pharmacological treatments, including antibacterial aminoglycoside antibiotics, anticancer agents, and loop diuretics.

Material & Methods

We previously set Bovine Cochlear Pericytes (BCPs) as an in vitro model of stria vascularis. Here we evaluated the sensitivity of BCPs to ototoxic drugs, by challenging with furosemide, cisplatin and gentamicin (10-250 µM) for 24h and 48h. In some conditions, cells were also co-treated with ototoxic drug (250 µM) and dexamethasone (1-100 nM) for 48h to evaluate its potential protective effect. Cell viability was evaluated by MTT assay and LDH release. Reactive oxygen species (ROS) production was estimated by using 2',7'-dichlorofluoresceindiacetate (DCFDA). The percentage of apoptotic cells was assessed through cytofluorimetric analysis (Annexin V).
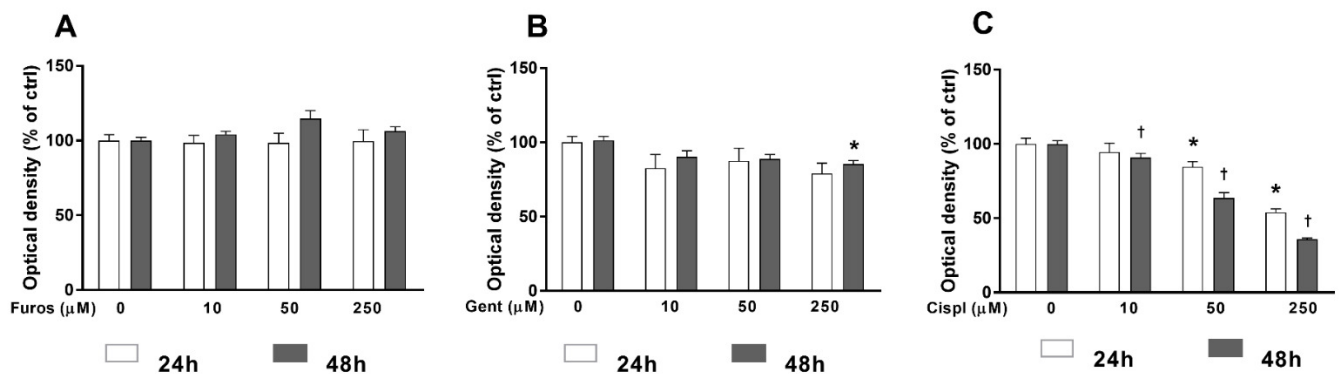

Fig.1:MTT assay; A: furosemide, at all tested concentration, did not decrease BCPs viability; **B:** gentamicin (250 µM) reduced cell viability in BCP after 48h of treatment. * $p < 0.05$ vs control (48h); **C:** cisplatin 250 µM led to major statistically significant reduction in cell viability after 48h of treatment. Values are reported as mean ± SEM of results from three different experiments. *$p < 0.05$ vs control (24h); † $p < 0.05$ vs control (48h)

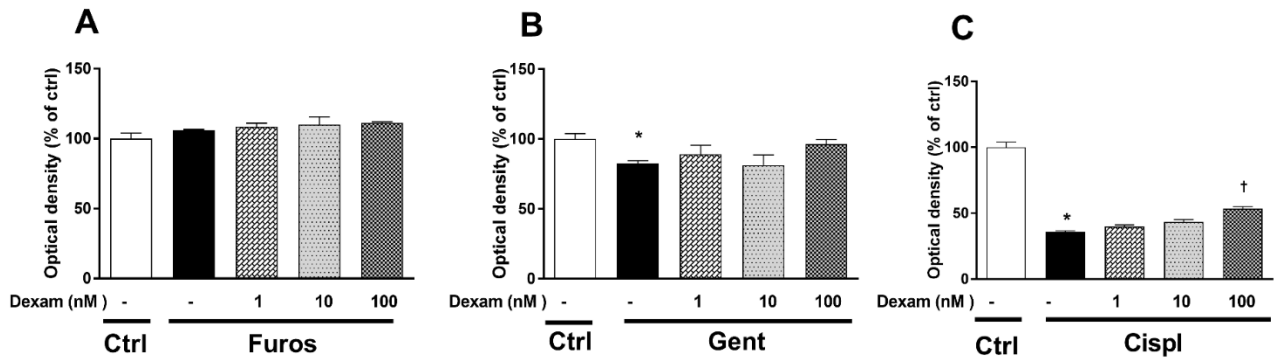**Fig.2: MTT assay co-treatment with ototoxic drugs (250 µM) and dexamethasone(1-100 nM) for 48h; A-B-C:** Dexamethasone 100 nM improved cell viability of cells exposed to gentamicin or cisplatin. Values are reported as mean ± SEM of results from three different experiments. *p< 0.05 vs control; † p< 0.05 vs Cisplatin without dexamethasone
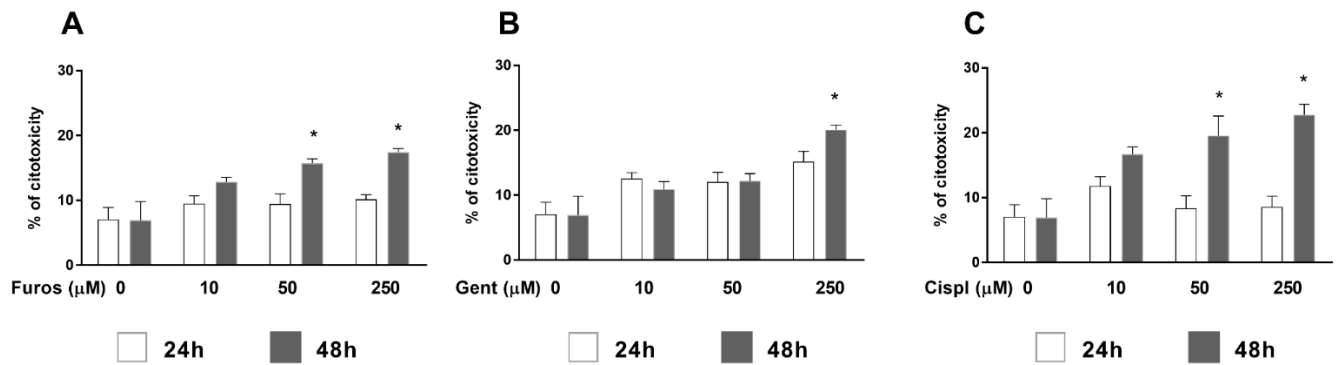

Fig.3: LDH assay; **A**: the release of LDH by BCP increased in a dose dependent manner after 48h treatment with furosemide (10-250 µM) **B**: Gentamicin 250µM led to greater statistically significant release of LDH after 48h of treatment. **C**: cisplatin (10-250 µM) caused LDH release in a dose dependent manner after 48h.

Values are reported as mean ± SEM of results from three different experiments. *p< 0.05 vs control(48h);
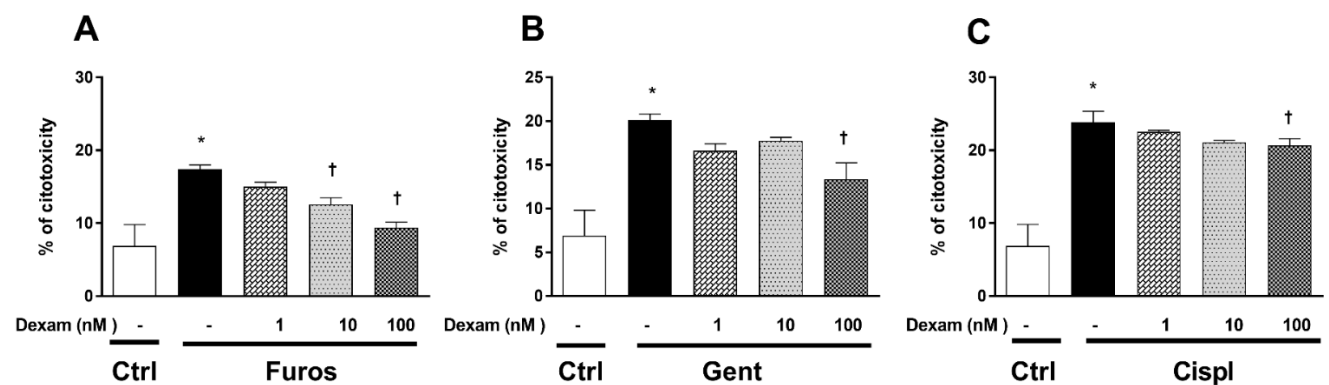
Fig.4: LDH assay co-treatment with ototoxic drugs (250 μM) and dexamethasone(1-100 nM) for 48h: A-B-C: dexamethasone 100 nM significanlty decreased LDH release with all ototoxic drugs. Values are reported as mean ± SEM of results from three different experiments. *p< 0.05 vs control,
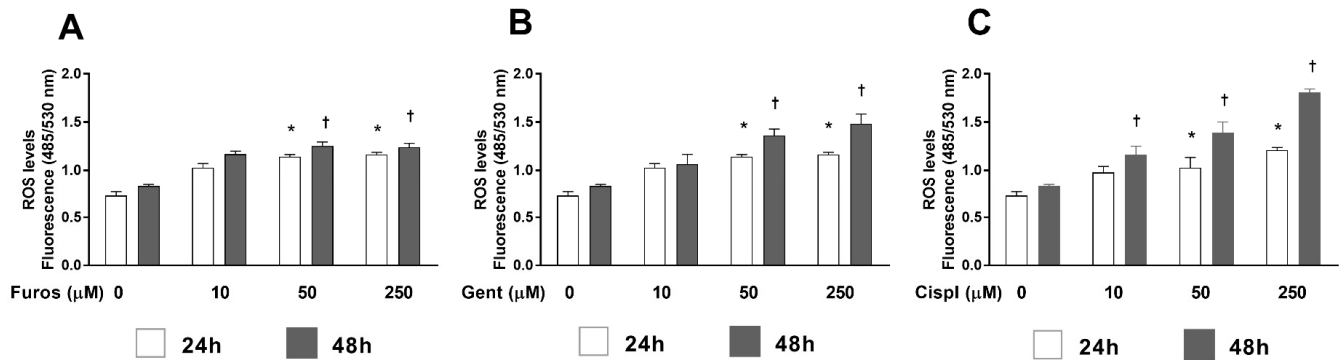† p< 0.05 vs ototoxic drugs without dexamethasone

Fig.5: ROS production: A-B-C: Furosemide, gentamicin and cisplatin significantly increased ROS production by BCPs, expecially at concentration of 250 μM after 48h. Values are reported as mean ± SEM of results from three different experiments. *p< 0.05 vs control (24h), † p< 0.05 vs control (48h).
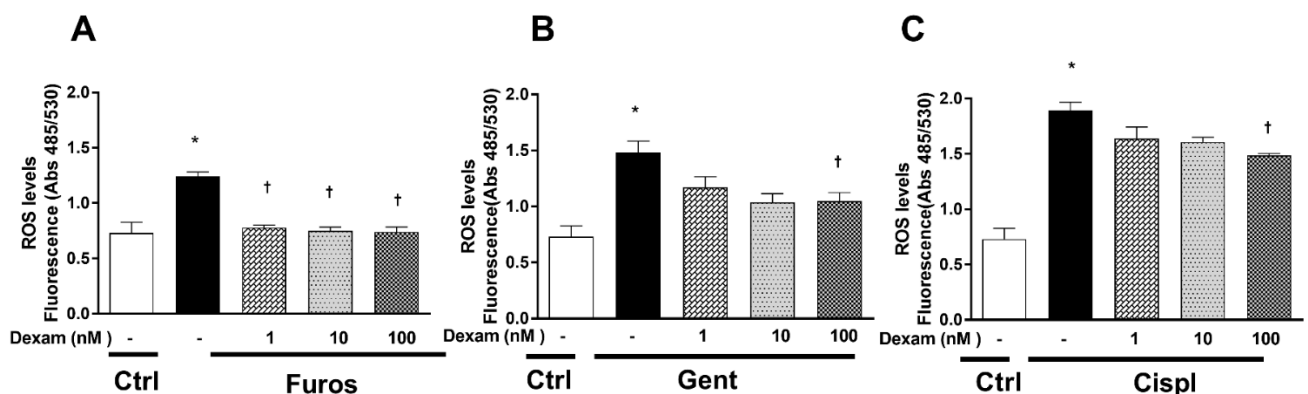

Fig.6: ROS production co-treatment with ototoxic drugs (250 μM) and dexamethasone(1-100 nM) for 48h: A: dexamethasone decreased furosemide-induced ROS production already at 1 nM. **B-C:** dexamethasone 100 nM significantly decreased ROS production induced by gentamicin and cisplatin 250μM. Values are reported as mean ± SEM of results from three different experiments. *p< 0.05 vs control, † p< 0.05 vs ototoxic drugs without dexamethasone.
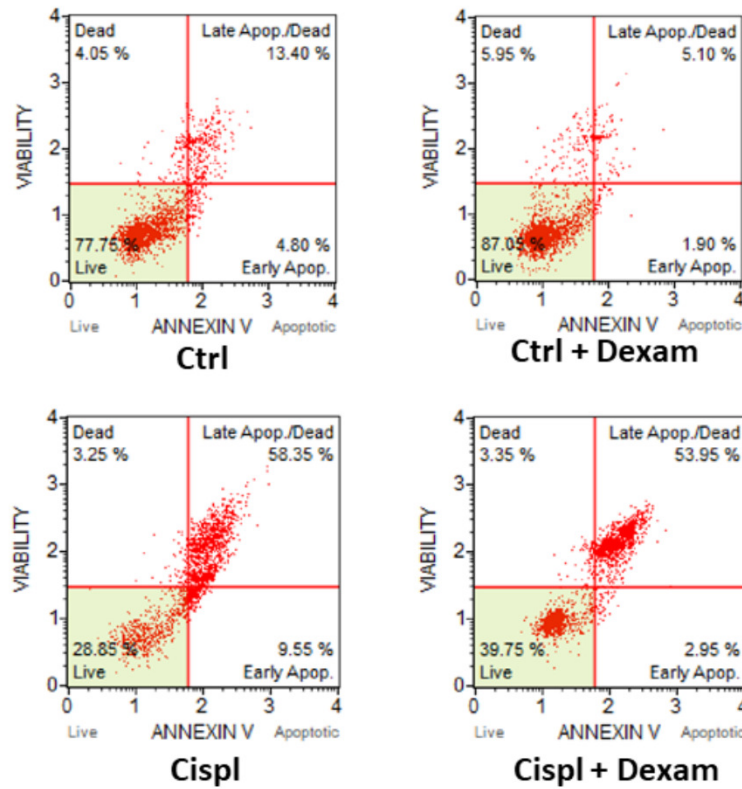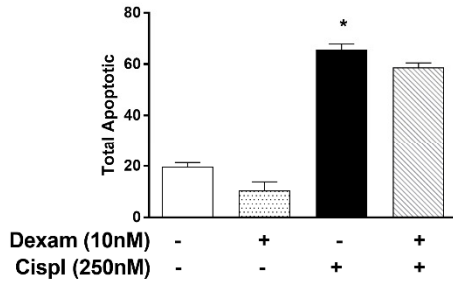
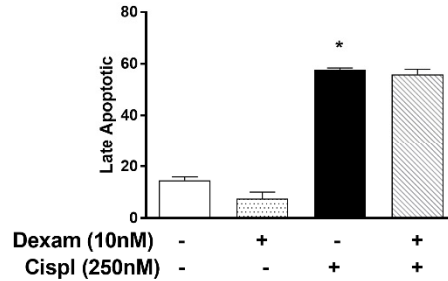**Fig.7: Annexin V test**: **A:** representative plots of cell populatin analyzed **B**: total apoptotic cells**; C:** late apoptotic cells**;** D:early apoptotic cells**;-E**: live cells. Dexamethasone 100 nM decreased the percentage of apoptotic BCPs teatred with cisplatin 250 µM. Values are reported as mean ± SEM of results from three different experiments. *p< 0.05 vs control, † p< 0.05 vs cisplatin.
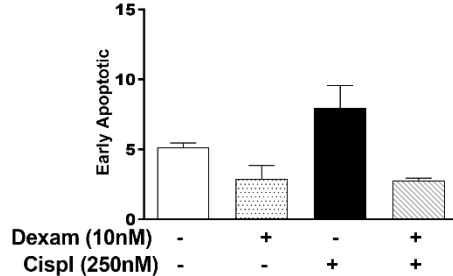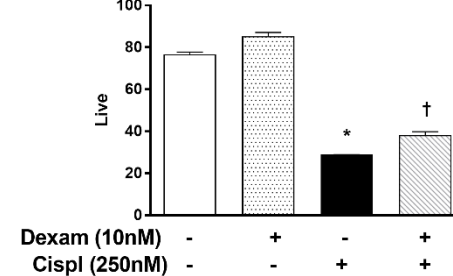
Results

We observed a significant reduction of cell viability (about 15%) in BCP treated with gentamicin (250 µM) for 48h, while furosemide, at all tested concentration, did not decrease BCPs viability. Treatment with cisplatin reduced cell viability by about 15% and 50% after 24h with 50 µM and 250 µM respectively ($p < 0.05$) and by about 15%, 35% and 65% after 48h of treatment with 10 µM, 50 µM and 250 µM respectively ($p < 0.05$) compared to control. Dexamethasone (100 nM) improved cell viability ($p < 0.05$) of cells exposed to gentamicin or cisplatin. The release of LDH by BCPs increased in a dose dependent manner ($p < 0.05$) after a 48h treatment with furosemide, cisplatin, or gentamicin. Co-treatment with dexamethasone (100 nM) significantly decreased LDH release with all ototoxic drugs. Furosemide, gentamicin and cisplatin significantly increased ROS production by BCPs, especially at concentration of 250 µM after 48h, while co-treatment with dexamethasone (100 nM) significantly ($p < 0.05$) decreased ROS production. Worthy of note, dexamethasone decreased furosemide-induced ROS production already at 1 nM. Finally, dexamethasone 100 nM decreased the percentage of apoptotic BCPs treated with cisplatin 250 µM.

Discussion and Conclusions

We validated a new in-vitro model of stria vascularis by assessing the effects of known ototoxic drugs on different endpoints. The results indicate that this in vitro model of stria vascularis may serve as a tool in pharmacologic and toxicodynamic preclinical studies, to screen ototoxic effects of new chemical entities and/or drugs endowed with putative protective action to prevent hearing loss.

11 General Discussion and Conclusions

The functions of endothelial-blood/tissue barrier is critical for maintaining and controlling tissue homeostasis and protecting neuronal tissue against the infiltration of harmful substances and to regulate the permeation of beneficial endogenous substances between the blood and the extracellular fluid of the brain. The blood-labyrinth barrier in the *stria vascularis* is anatomically different from blood barriers in the retina. To study the physiology of these biological dynamic separations, still largely unknown, *in vitro* cell-culture models can be used. In the barrier the PCs are involved in vascular development, integrity, angiogenesis and tissue fibrogenesis; their functions are linked to the regulation of

cerebral blood flow, barrier permeability and vascular formation and may be disrupted by neuroinflammation. Understanding the role of PCs in pathophysiology, particularly how it relates to microvascular degeneration and pathological neovascularisation, is critical in order to develop novel therapies to complement or improve current treatment options. The loss of PCs in the retina has been recently linked to the breakdown of the BRB and to the infiltration of inflammatory cells in the DR. Current therapies can only tone down the progression of the disease; stem cell-based approaches may represent a valid alternative to limit pericyte loss in DR. In particular adipose-derived MSCs were investigated for their multipotent differentiation ability and used as a tool for potential therapeutic applications in DR. In our study, the potential of adipose stem cells (ASCs) in differentiating into the phenotype of PCs was evaluated by immuno-analysis and the effects of ASC like-pericytes on hRECs on barrier formation was examined by assessing the levels of junction proteins and by measuring TEER. ASCs cultured in the pericyte medium with Pericyte Growth Supplement (PGS) was both positive to α-SMA and NG$_2$, similary to hRPCs. Western blot analysis confirmed that the expression of markers in the ASCs treated with PGS were comparable to that of hRPCs. Measuring TEER showed that the ASCs pre-treated with PGS, similar to hRPCs, increased the integrity of the hREC membrane. The immunocytochemical investigation of junctional proteins, typical of BRB, showed more organization on the endothelial cell membrane in co-culture with ASCs treated with PGS, than with other co-cultures. Supporting data also came from experiments of three-dimensional co-cultures, where a comparable phenotype was observed for hRPCs and hASCs treated with PGS. Overall, the present results indicate that a closer pericyte-like differentiation can be achieved when hASCs are grown in a culture medium specifically designed for PCs. Among the PGS components present in pericyte medium, Fibroblast Growth Factor-2 (FGF-2) is probably responsible for these effects. In fact, it has been reported that FGF-2 maintains cultured PCs in a proliferative state, characterized by low levels of α-SMA; (Papetti et. al, 2003) consequently, the absence of FGF-2 induces a pericyte switch from a proliferative to a contractile phenotype. Thus, it can be assumed that these pericyte-like differentiated hASCs may mimic the functional role normally played by PCs in strengthening the BRB. Altogether, immunocytochemical findings would indicate that pericyte-like hASCs may represent a candidate for a cell-based therapy aimed at reconstructing a damaged BRB. In fact, when pericyte-like ASCs, not yet expressing the filamentous α-SMA pattern, are still in the proliferative stage, they are most suitable to interact with ECs to build up an efficient

BRB. Therefore, ASC-based therapeutic approaches may be usefully exploited to restore retinal microvasculature integrity.

PCs also play a role in other neurovascular units, such as the *stria vascularis*; even in these barriers, PCs are linked to regulating cerebral blood flow, barrier permeability, cerebral vascular formation maintenance and neuroinflammation. The *stria vascularis* is also known as the blood-labyrinth barrier and is structurally and functionally similar to the blood-brain barrier and its dysruption plays an important role in hearing loss following acoustic trauma and drug-related ototoxicity. Many methods of isolation and culture of barrier cells from different tissues are currently available but isolating cells from the vestibular system is still difficult and labor intensive. It has recently been shown that PCs are particularly susceptible to proinflammatory stimuli; for example, the reduction of the precapillary diameter, which strongly contributes to blood flow impairment in the *stria vascularis*, is related to PC rearrangement induced by TNF. The *stria vascularis* is the target of damage induced by a variety of drugs, the normal function of the stria is crucial to maintain the ionic gradients and endocochlear potential (EP). In the study, we described a novel growth medium-based method associated with a mild enzymatic digestion to obtain primary PCs from the *stria vascularis* of bovine cochlea explants. Furthermore, we evaluated the sensitivity of primary bovine cochlear BPCs to three potentially ototoxic drugs: furosemide, gentamicin and cisplatin, at different concentrations. We obtained high-quality PCs from the cochlea isolated from a just slaughtered bovine animal and immediately placed in colture. The isolated BCPs were characterized by immunostaining of PC markers: the BCPs, like hRPCs, are immunopositive for PDGFR, NG^2 and α-SMA, but they do not express the von Willebrand factor (vWF), a typical marker of ECs. We validated a new *in vitro* model of the *stria vascularis* by assessing the effects of known ototoxic drugs. The effects of cisplatin and gentamicin on the viability of hRECs, BCPs, hRPCs were evaluated to verify the susceptibility of cochlear cells to ototoxic drugs, compared to other cell lines. The test showed that the greatest effect is on BCPs. MTT viability test, cytotoxicity test by LDH production, detection of oxidative stress through the investigation of total ROS by the 2',7'-dichlorofluorescin diacetate (DCFDA) method and MUSE cytofluorimetric analysis of the Annexin V apoptotic test were used to verify the susceptibility of cochlear cells to ototoxic drugs. The data showed the susceptibility of BCPs to the ototoxic drugs that the damage was dose and time dependent and demonstrated the greater cytotoxic potential of cisplatin. The use of anti-inflammatories, such as dexamethasone, slightly reduced

the toxic effects of the drugs. The results indicate that this *in vitro* model of the *stria vascularis* may serve as a tool in pharmacologic and toxicodynamic preclinical studies, to screen ototoxic effects of new chemical compounds and/or drugs endowed with a putative protective action to prevent hearing loss. The use of BCPs for the analysis of ototoxic drugs can be used as a model to investigate pharmacological mechanisms and protection systems against different insults.

General list of References (general introduction and concluding remarks)

- Alharazneh, A. et al. (2011). Functional hair cell mechanotransducer channels are required for aminoglycoside ototoxicity. PLoS ONE 6, e22347.
- Allt G., Lawrenson JG. Reta Lila Weston, (2001). Pericytes: cell biology and pathology. Cells Tissues Organs. 169(1):1-11. Inst. of Neur. Studies, U. C. L. M. S., London, UK.
- Armulik A, Genove G, Betsholtz C, (2011). Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell 21:193–215
- Baynes, J.W. (1991). Role of oxidative stress in development of complications in diabetes. Diabetes. 40, 405412.
- Bazzoni G, Martinez-Estrada OM, Orsenigo F, Cordenonsi M, Citi S, Dejana E., (2000). Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. J Biol Chem.
- Behl, Tapan et al., (2016). Implication of oxidative stress in progression of diabetic retinopathy." Survey of ophthalmology 61 2, 187-96 .
- Beltramo, E.; Berrone, E.; Tarallo, S.; Porta, M., (2008). Effects of thiamine and benfotiamine on intracellular glucose metabolism and relevance in the prevention of diabetic complications. Acta Diabetol. 45, 131-141.
- Bertlich, M., Ihler, F., Weiss, B. G., Freytag, S., Strupp, M., & Canis, M., (2017). Cochlear pericytes are capable of reversibly decreasing capillary diameter in vivo after tumor necrosis factor exposure. Otology & Neurotology, 38, e545–e550
- Birbrair A, Sattiraju A, Zhu D, Zulato G, Batista I, Nguyen VT, Messi ML, Solingapuram Sai KK, Marini FC, Delbono O, Mintz A, (2017). Novel Peripherally Derived Neural-Like Stem Cells as Therapeutic Carriers for Treating Glioblastomas. Stem Cells Transl Med. doi: 10.5966/sctm.2016-0007. Epub 2016 Sep 14

- Bisht M, Bist SS., (2011). Ototoxicity: the hidden menace. Indian Journal of Otolaryngology and Head and Neck Surgery : Official Publication of the Association of Otolaryngologists of India. 63(3):255-259. DOI: 10.1007/s12070-011-0151-8.

- Bisht, Manisha, and S S Bist, (2011). Ototoxicity: the hidden menace. Indian journal of otolaryngology and head and neck surgery : official publication of the Association of Otolaryngologists of India vol. 63,3: 255-9. doi:10.1007/s12070-011-0151-8

- Bourin, P.; Bunnell, B.A.; Casteilla, L.; Dominici, M.; Katz, A.J.; March, K.L.; Redl, H.; Rubin, J.P.; Yoshimura, K.; Gimble, J.M., (2013). Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: A joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy, 15, 641–648. [PubMed]

- Breglio, A.M., Rusheen, A.E., Shide, E.D. et al., (2017). Cisplatin is retained in the cochlea indefinitely following chemotherapy. Nat Commun 8, 1654. doi.org/10.1038/s41467-017-01837-1

- Brownlee M., (2005). The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 54:1615-25.

- Brownlee Michael, (2005). The Pathobiology of Diabetic Complications. A Unifying Mechanism. Diabetes. doi.org/10.2337/diabetes.54.6.1615

- Brownlee, M., (2001). Biochemistry and molecular cell biology of diabetic complications. Nature. 414, 813-820.

- Canis M, Bertlich M., (2019). Cochlear Capillary Pericytes. Adv Exp Med Biol. doi: 10.1007/978-3-030-11093-2_7.

- Carmeliet P., Lampugnani M.G., Moons L., Breviario F., Compernolle V., Bono F., et al., (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell, 98 (2), pp. 147-157

- Chen MS, Kao CS, Fu CC, Chen CJ, Tai TY., (1995). Incidence and progression of diabetic retinopathy among non-insulin-dependent diabetic subjects: a 4-year follow-up. Int J Epidemiol. 24:787–795.

- Choi, Yoon Kyung and Kyu-Won Kim. (2008). Blood-neural barrier: its diversity and coordinated cell-to-cell communication. BMB reports 41 5: 345-52 .

- Close S. Hori, S. Ohtsuki, K. Hosoya, E. Nakashima, T. Terasaki, (2004). A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. J. Neurochem.

- Dai, C. F., & Steyger, P. S., (2008). A systemic gentamicin pathway across the *stria vascularis*. Hearing research, 235(1-2), 114–124. doi:10.1016/j.heares.2007.10.010

- Dasari, S. & Tchounwou, P. B., (2014).Cisplatin in cancer therapy: molecular mechanisms of action. Eur. J. Pharmacol. 740, 364–378.

- Díaz-Coránguez Mónica, Ramos Carla, Antonetti David A., (2017). The inner blood-retinal barrier: Cellular basis and development. Vision Research. Vol. 139, 123-137 doi.org/10.1016/j.visres.2017.05.009

- Ding D., Salvi R., (2005). Review of cellular changes in the cochlea due to aminoglycoside antibiotics Volta Rev., 105, pp. 407-438

- Ding, D., Allman, B.L., Salvi, R., (2012). Review: ototoxic characteristics of platinum antitumor drugs. Anat. Rec. 295, 1851e1867.

- Ding, D., Liu, H., Qi, W., Jiang, H., Li, Y., Wu, X., and Salvi, R., (2016). Ototoxic effects and mechanisms of loop diuretics. Journal of otology, 11(4), 145–156. doi:10.1016/j.joto.2016.10.001

- Ding, Dalian et al., (2016). Ototoxic effects and mechanisms of loop diuretics." Journal of otology vol. 11,4: 145-156. doi:10.1016/j.joto.2016.10.001

- Dohgu S., Takata F., Yamauchi A., Nakagawa S., Egawa E., Naito M., Tsuruo T., Sawada Y., Niwa M., Kataoka Y., (2005). Brain pericytes contribute to the induction and up-regulation of blood–brain barrier functions through transforming growth factor-beta production. Brain Res.

- Dohgu S., Yamauchi A., Takata F., Naito M., Tsuruo T., Higuchi S., Sawada Y., Kataoka Y., (2004).Transforming growth factor-beta1 upregulates the tight junction and P-glycoprotein of brain microvascular endothelial cells Cell. Mol. Neurobiol.

- Dominici, M.L.B.K.; Le Blanc, K.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.C.; Krause, D.S.; Deans, R.J.; Keating, A.; Prockop, D.J.; Horwitz, E.M., (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 8, 315–317

- Dore-Duffy P., Cleary K. (2011) Morphology and Properties of Pericytes. In: Nag S. (eds) The Blood-Brain and Other Neural Barriers. Methods in Molecular Biology (Methods and Protocols), vol 686. Humana Press

- Duport, S., Robert, F., Muller, D., Grau, G., Parisi, L., & Stoppini, L., (1998). An in vitro blood-brain barrier model: Cocultures between endothelial cells and organotypic brain slice cultures. Proceedings of the National Academy of Sciences of the United States of America, 95, 1840–1845.

- Eilken, H.M., Diéguez-Hurtado, R., Schmidt, I. et al., (2017). Pericytes regulate VEGF-induced endothelial sprouting through VEGFR1. Nat Commun 8, 1574. doi.org/10.1038/s41467-017-01738-3

- Ejaz S1, Chekarova I, Ejaz A, Sohail A, Lim CW, (2008). Importance of pericytes and mechanisms of pericyte loss during diabetes retinopathy. Diabetes Obes Metab. 10(1):53-63. Epub 2007 Oct 15.

- Farquhar M. G., G.E. Palade, (1963). Junctional complexes in various epithelia. Journal of Cell Biology.

  FEBS Letters, Volume 546, Issue 1, Pages 154-158.

- Fisher, M., (2009). Pericyte signaling in the neurovascular unit. Stroke, 40(3 Suppl), S13–S15.

- Franz, P., Helmreich, M., Stach, M., Franz-Italon, C., Bock, P., (2004). Distribution of actin and myosin in the cochlear microvascular bed. Acta Otolaryngol. 124, 481e485.

- Frey T, Antonetti DA., (2011). Alterations to the blood-retinal barrier in diabetes: cytokines and reactive oxygen species. Antioxid Redox Signal.

- Gabbay KH, Merola LO, Field RA, (1966). Sorbitol pathway: presence in nerve and cord with substrate accumulation in diabetes. Science 151: 209–210

- Gale GR, Rosenblum MG, Atkins LM, Walker EM Jr, Smith AB, Meischen SJ. (1973). Antitumor action of cis-dichlorobis(methylamine)platinum(II). J. Natl Cancer Inst. 51, 1227–1234.

- Ganesan, Purushothaman et al., (2018). Ototoxicity: A Challenge in Diagnosis and Treatment." Journal of audiology & otology vol. 22,2. 59-68. doi:10.7874/jao.2017.00360

- Ganesan, Purushothaman et al., (2018). Ototoxicity: A Challenge in Diagnosis and Treatment. Journal of audiology & otology vol. 22,2; 59-68. doi:10.7874/jao.2017.00360

- Gardner TW, Abcouwer SF, Barber AJ, Jackson GR., (2011). An Integrated Approach to Diabetic Retinopathy Research. Arch Ophthalmol. 129(2):230–235. doi:10.1001/archophthalmol.2010.362

- Gentilin E, Simoni E, Candito M, Cazzador D, Astolfi L., (2019). Cisplatin-Induced Ototoxicity: Updates on Molecular Targets. Trends Mol Med.; 25(12):1123-1132. doi: 10.1016/j.molmed.2019.08.002.

- Giardino, I.; Edelstein, D.; Brownlee, M., (1994). Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters basic fibroblast growth factor activity. A model for intracellular glycosylation in diabetes. J. Clin. Invest. 94, 110-117.

- Giurdanella G., Anfuso C. D., Olivieri M., Lupo G., Caporarello N., Eandi C. M. , Drago F., Bucolo C., Salomone S., (2015). Aflibercept, bevacizumab and ranibizumab prevent glucose-induced damage in human retinal pericytes in vitro, through a PLA2/COX-2/VEGF-A pathway. Biochemical Pharmacology, Volume 96, Issue 3, Pages 278-287

- Greenwood G. J., (1959). Neomycin ototoxicity; report of a case. A.M.A. Archives of Otolaryngology, vol. 69, no. 4, pp. 390–397.

- Guo J., Chai R., Li H., Sun S., (2019) Protection of Hair Cells from Ototoxic Drug-Induced Hearing Loss. In: Li H., Chai R. (eds) Hearing Loss: Mechanisms, Prevention and Cure. Advances in Experimental Medicine and Biology, vol 1130. Springer, Singapore

- Gupta MP, Herzlich AA, Sauer T, Chan CC., (2015). Retinal Anatomy and Pathology. Dev Ophthalmol. 55:7-17. doi: 10.1159/000431128

- Gupta MP, Herzlich AA, Sauer T, Chan CC., (2015). Retinal Anatomy and Pathology. Dev Ophthalmol. 2016;55:7-17. doi: 10.1159/000431128.

- Harrell CR, Markovic BS, Fellabaum C, Arsenijevic A, Djonev V, Volarevic V (2018) Molecular mechanisms underlying therapeutic potential of pericytes. J Biomed Sci 25:21

- Hibino, H., Nin, F., Tsuzuki, C., & Kurachi, Y., (2010). How is the highly positive endocochlear potential formed? The specific architecture of the *stria vascularis* and the roles of the ion-transport apparatus. Pflugers Archiv: European Journal of Physiology, 459, 521–533.

- Hirase T, Staddon JM, Saitou M, Ando-Akatsuka Y, Itoh M, Furuse M, Fujimoto K, Tsukita S, Rubin LL., (1997). Occludin as a possible determinant of tight junction permeability in endothelial cells. J. Cell Sci.

- Hori S, Ohtsuki S, Hosoya K, Nakashima E, Terasaki T., (2004). A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. J. Neurochem.

- Hudspeth, A. J., (2005). How the ear's works work: mechanoelectrical transduction and amplification by hair cells. Comptes rendus biologies 328 2: 155-62 .

- Ismail-Beigi F., Craven T., Banerji M. A., et al., (2010). Effect of intensive treatment of hyperglycaemia on microvascular outcomes in type 2 diabetes: an analysis of the ACCORD randomised trial. The Lancet. 376(9739):419–430. doi: 10.1016/S0140-6736(10)60576-4.

- Itoh M, Furuse M, Morita K, Kubota K, Saitou M, Tsukita S., (1999). Direct binding of three tight junctionassociated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. J Cell Biol.

- Itoh M, Nagafuchi A, Moroi S, Tsukita S., (1997). Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. J Cell Biol. 138(1):181–192.

- Jenkins AJ, Joglekar MV, Hardikar AA, et al., (2015). Biomarkers in diabetic retinopathy. Rev Diabet Stud.12:159.

- Karasawa, T., Sibrian-Vazquez, M., Strongin, R. M. & Steyger, P. S., (2013). Identification of cisplatin-binding proteins using agarose conjugates of platinum compounds. PLoS ONE 8, 66220.

- Kim, Jung Mo et al. (2016). Perivascular Progenitor Cells Derived From Human Embryonic Stem Cells Exhibit Functional Characteristics of Pericytes and Improve the Retinal Vasculature in a Rodent Model of Diabetic Retinopathy. Stem cells translational medicine vol. 5,9: 1268-76. doi:10.5966/sctm.2015-0342

- Knight, Kristin & Kraemer, Dale & Neuwelt, Edward, (2006). Ototoxicity in Children Receiving Platinum Chemotherapy: Underestimating a Commonly Occurring Toxicity That May Influence Academic and Social Development. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 23. 8588-96. 10.1200/JCO.2004.00.5355..

- Kolm-Litty V, Sauer U, Nerlich A, Lehmann R, Schleicher ED, (1998). High glucose-induced transforming growth factor beta1 production is mediated by the hexosamine pathway in porcine glomerular mesangial cells. J Clin Invest 101: 160–169.

- Lai, C. H., & Kuo, K. H., (2005). The critical component to establish in vitro BBB model: Pericyte. Brain Research Reviews, 50, 258–265.

- Lee Ann Remington OD, (2012). Clinical Anatomy and Physiology of the Visual System. (Third Edition),Textbooks

- Lee, A.Y.; Chung, S.S., (1999). Contributions of polyol pathway to oxidative stress in diabetic cataract. FASEB J.13, 23-30.

- Lelkes P.I., Hahn K.L., Sukovich D.A., Karmiol S., Schmidt D.H., (1998). On the Possible Role of Reactive Oxygen Species in Angiogenesis. In: Hudetz A.G., Bruley D.F. (eds) Oxygen Transport to Tissue XX. Advances in Experimental Medicine and Biology, vol 454. Springer, Boston, MA

- Liu Q, Yang Y, Fan X. (2020). Microvascular pericytes in brain-associated vascular disease. Biomed Pharmacother. doi: 10.1016/j.biopha.2019.109633. Epub 2019 Nov 25.

- Liu, Hong et al., (2011). Ototoxic destruction by co-administration of kanamycin and ethacrynic acid in rats. Journal of Zhejiang University. Science. B vol. 12,10: 853-61. doi:10.1631/jzus.B1100040

- Liu, T.Y.A., Arevalo, J.F., (2019). Wide-field imaging in proliferative diabetic retinopathy. Int J Retin Vitr 5, 20 doi:10.1186/s40942-019-0170-2.

- Lo Furno D, Mannino G, Cardile V, Parenti R, Giuffrida R., (2016). Potential Therapeutic Applications of Adipose-Derived Mesenchymal Stem Cells. Stem Cells and Development.. (21):1615-1628. DOI: 10.1089/scd.2016.0135.

- Lo Furno, D, Mannino, G, Giuffrida, R., (2018). Functional role of mesenchymal stem cells in the treatment of chronic neurodegenerative diseases. J Cell Physiol. 233: 3982– 3999. doi.org/10.1002/jcp.26192

- Locher, Heiko et al., (2015). Development of the *stria vascularis* and potassium regulation in the human fetal cochlea: Insights into hereditary sensorineural hearing loss. Developmental neurobiology vol. 75,11; 1219-40. doi:10.1002/dneu.22279

- Marcotti W., Van Netten S.M., C. J. Kros, (2005). The aminoglycoside antibiotic dihydrostreptomycin rapidly enters mouse outer hair cells through the mechano-electrical transducer channels. Journal of Physiology, vol. 567, no. 2, pp. 505–521.

- Marcus, Daniel & Wu, Tao & Wangemann, Philine & Kofuji, Paulo. (2002). KCNJ10 (Kir4.1) potassium channel knockout abolishes endocochlear potential. American journal of physiology. Cell physiology. 282. C403-7. 10.1152/ajpcell.00312.2001.

- Marshak T., Steiner M., Kaminer M., Levy L., Shupak A.; (2014). Prevention of cisplatin-induced hearing loss by intratympanic dexamethasone: a randomized controlled study Otolaryngol. Head Neck Surg., 150, pp. 983-990

- McPherson D. R., (2018). Sensory Hair Cells: An Introduction to Structure and Physiology. Integrative and comparative biology, 58(2), 282–300. doi:10.1093/icb/icy064

- Neri, S., (2019). Genetic Stability of Mesenchymal Stromal Cells for Regenerative Medicine Applications: A Fundamental Biosafety Aspect. Int. J. Mol. Sci., 20, 2406.

- Ohtsuki S, Yamaguchi H, Katsukura Y, Asashima T, Terasaki T., (2008). mRNA expression levels of tight junction protein genes in mouse brain capillary endothelial cells highly purified by magnetic cell sorting. J Neurochem.

- Ono Y, Aoki S, Ohnishi K, et al., (1998). Increased serum levels of advanced glycation end-products and diabetic complications. Diabetes Research and Clinical Practice..41(2):131-137. DOI: 10.1016/s0168-8227(98)00074-6.

- Palumbo, P.; Lombardi, F.; Siragusa, G.; Cifone, M.G.; Cinque, B.; Giuliani, M., (2018). Methods of Isolation, Characterization and Expansion of Human Adipose-Derived Stem Cells (ASCs): An Overview. Int. J. Mol. Sci., 19, 1897

- Papetti, Michael & Shujath, Jaleel & Riley, Kathleen & Herman, Ira, (2003). FGF-2 antagonizes the TGF-beta1-mediated induction of pericyte alpha-smooth muscle actin expression. Investigative ophthalmology & visual science. 44. 4994-5005.

- Park DY, Lee J, Kim J, Kim K, Hong S, Han S et al., (2017). Plastic roles of pericytes in the blood-retinal barrier. Nat Commun. 8: 15296.

- Patuzzi, Robert., (2011). Ion flow in *stria vascularis* and the production and regulation of cochlear endolymph and the endolymphtic potential. Hearing research. 277. 4-19. 10.1016/j.heares.2011.01.010.

- Pfister, F.; Lin, J.; Hammes, H.P., (2010). Pericyte Loss in the Diabetic Retina. In: Experimental Approaches to Diabetic Retinopathy; Hammes, H.P.; Porta, M. Eds.; Front Diabetes, Karger: Basel. Vol. 20, pp. 61–78.

- Purves, D., Augustine GJ, Fitzpatrick D, et al. (2001) Neuroscience. 2nd Edition, Sinauer Associates, Sunderland, MA.

- Quraishi, I. H., & Raphael, R. M., (2008). Generation of the endocochlear potential: A biophysical model. Biophysical Journal, 94, L64–L66.

- Romero-Aroca, P., Baget-Bernaldiz, M., Pareja-Rios, A., Lopez-Galvez, M., Navarro-Gil, R., & Verges, R., (2016). Diabetic Macular Edema Pathophysiology: Vasogenic versus Inflammatory. Journal of diabetes research, 2156273. doi:10.1155/2016/2156273

- Romero-Aroca, Pedro & Baget-Bernaldiz, Marc & Ríos, Alicia & Lopez-Galvez, Maribel & Navarro-Gil, Raul & Verges, Raquel. (2016). Diabetic Macular Edema Pathophysiology: Vasogenic versus Inflammatory. J Diabetes Res. 10.1155/2016/2156273.

- Rybak LP, (1993). Ototoxicity of loop diuretics. Otolaryngol Clin North Am., 26 (5):829-44.

- Rybak, L.P. (1985), Furosemide ototoxicity: Clinical and experimental aspects. The Laryngoscope, 95: 1-14. doi:10.1288/00005537-198509010-00001

- Sabol, R.A.; Bowles, A.C.; Côté, A.; Wise, R.; Pashos, N.; Bunnell, B.A., (2018). Therapeutic Potential of Adipose Stem Cells. Adv. Exp. Med. Biol.

- Salt, A. N., Melichar, I., & Thalmann, R., (1987). Mechanisms of endocochlear potential generation by *stria vascularis*. The Laryngoscope, 97, 984–991.

- Salt, A.N., Mleichar, I. and Thalmann, R., (1987), Mechanisms of endocochlear potential generation by *stria vascularis*. The Laryngoscope, 97: 984-991. doi:10.1288/00005537-198708000-00020

- Schacht J., et al. (2012). Cisplatin and aminoglycoside antibiotics: hearing loss and its prevention Anat. Rec. (Hoboken), 295, pp. 1837-1850

- Schellack Natalie & Naude Alida, (2013). An overview of pharmacotherapy-induced ototoxicity, South African Family Practice, 55:4, 357-365, DOI: 10.1080/20786204.2013.10874377

- Selimoglu E., (2007). Aminoglycoside-induced ototoxicity .Curr Pharm Des.,13(1):119-26..

- Shepro, D., & Morel, N. M., (1993). Pericyte physiology. FASEB Journal. 7(11), 1031–1038.

- Shi X, (2016). Pathophysiology of the cochlear intrastrial fluid-blood barrier (review). Hearing research, 338, 52–63. doi:10.1016/j.heares.

- Shi, X., (2009). Cochlear pericyte responses to acoustic trauma and the involvement of hypoxia-inducible factor-1alpha and vascular endothelial growth factor. Am.J. Pathol. 174, 1692e1704.

- Shi, X., (2016). Pathophysiology of the cochlear intrastrial fluid-blood barrier. Hearing Research, 338, 52–63.

- Shi, Xiaorui et al., (2008). The cochlear pericytes. Microcirculation (New York, N.Y.: 1994) vol. 15,6: 515-29. doi:10.1080/10739680802047445

- Shi, Xiaorui, (2011). Physiopathology of the cochlear microcirculation. Hearing research vol. 282,1-2: 10-24. doi:10.1016/j.heares.2011.08.006

- Spencer, B.G., Estevez, J.J., Liu, E. et al. (2019). Pericytes, inflammation, and diabetic retinopathy. Inflammopharmacol. doi.org/10.1007/s10787-019-00647-9

- Steel, Karen P and Christopher Barkway, (1989). Another role for melanocytes: their importance for normal *stria vascularis* development in the mammalian inner ear. Development 107 3: 453-63.

- Steyger, Peter S et al., (2018). Editorial: Cellular Mechanisms of Ototoxicity. Frontiers in cellular neuroscience vol. 12 75. 27, doi:10.3389/fncel.2018.00075

- Stitt AW, Lois N, Medina RJ et al., (2013). Advances in our understanding of diabetic retinopathy. Clin Sci (Lond). 25:1–17.

- Sun, N., Panetta, N. J., Gupta, D. M., Wilson, K. D., Lee, A., Jia, F., Wu, J. C., (2009). Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. Proceedings of the National Academy of Sciences of the United States of America, 106(37), 15720–15725. doi:10.1073/pnas.0908450106

- Sweeney MD, Sagare AP, Zlokovic BV., (2018). Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. Nat Rev Neurol.

- Tabuchi K, Nishimura B, Nakamagoe M, et al., (2011). Ototoxicity: mechanisms of cochlear impairment and its prevention. Current Medicinal Chemistry. 18(31):4866-4871. DOI: 10.2174/092986711797535254.

- Takeuchi S, Ando M, Sato T, Kakigi A. Hear Res., (2001). Three-dimensional and ultrastructural relationships between intermediate cells and capillaries in the gerbil *stria vascularis*

- Takeuchi, S., Ando, M., Sato, T., & Kakigi, A. (2001). Three-dimensional and ultrastructural relationships between intermediate cells and capillaries in the gerbil *stria vascularis*. Hearing Research, 155, 103–112.

- Tam VH, Lam EP, Chu BC, Tse KK, Fung LM., (2009). Incidence and progression of diabetic retinopathy in Hong Kong Chinese with type 2 diabetes mellitus. J Diabetes Complications. 23:185–193.

- Tan GS, Cheung N, Simo R, Cheung GC, Wong TY., (2017). Diabetic macular oedema. Lancet Diabetes Endocrinol. 5:143–155.

- Ting KK1, Zhao Y1, Shen W2, Coleman P1, Yam M2, Chan-Ling T3, Li J1, Moller T4, Gillies M2, Vadas MA5, Gamble JR6, (2019). Therapeutic regulation of VE-cadherin

with a novel oligonucleotide drug for diabetic eye complications using retinopathy mouse models. Diabetologia. doi: 10.1007/s00125-018-4770-4. Epub 2018 Nov 15

- Tran Ba Huy, P., Bernard, P. & Schacht, J., (1986). Kinetics of gentamicin uptake and release in the rat. Comparison of inner ear tissues and fluids with other organs. J. Clin. Invest. 77, 1492–1500.

- Trost A, Lange S, Schroedi F, Bruckner D, Motloch KA, Bogner B, Kaser-Eichberger A, Strohmaier C, Runge C, Algner L, Rimera FJ, Reitsamer HA, (2016) Brain and retinal pericytes: origin, function and role. Frontiers Cell Neurosci 10:1–13

  Volume 1623, Pages 110-122.

- Vujosevic, Stela, & Caterina Toma., (2018). Diabetic retinopathy: an inflammatory disease. Annals of Eye Science [Online], 3.10

- Waguespack J. R. and Ricci A. J., (2014). Aminoglycoside ototoxicity: permeant drugs cause permanent hair cell loss. Journal of Physiology, vol. 567, no. 2, pp. 359–360.

- Wallez Y., Huber P., (2008). Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. Biochimica et Biophysica Acta (BBA) – Biomembranes Review. doi.org/10.1016/j.bbamem.2007.09.003

- Warboys CM, Fraser PA., (2010), Hyperglycemia attenuates acute permeability response to advanced glycation end products in retinal microvasculature. Microvascular Research. 174-176. DOI: 10.1016/j.mvr.2010.03.004.

- Weinstein M. J. , Luedemann G. M., Oden E. M. et al., (1963). Gentamicin, a new antibiotic complex from Micromonospora. Journal of Medicinal Chemistry, vol. 6, no. 4, pp. 463–464

- Wells Lance, Hart Gerald W., (2003). O-GlcNAc turns twenty: functional implications for post-translational modification of nuclear and cytosolic proteins with a sugar,

- Yue Yang, Min Dai, Teresa M. Wilson, Irina Omelchenko, John E. Klimek, Phillip A. Wilmarth, Larry L. David, Alfred L. Nuttall et al., (2011). Na^+/K^+-ATPase α1 Identified as an Abundant Protein in the Blood-Labyrinth Barrier That Plays an Essential Role in the Barrier Integrity. PLoS doi: 10.1371/journal.pone.0016547

- Yujie Chen, Qiang Li, Jiping Tang, Hua Feng, John H Zhang, (2015). The evolving roles of pericyte in early brain injury after subarachnoid hemorrhage.Brain Research,

- Zhang X, Zeng H, Bao S, et al., (2014). Diabetic macular edema: new concepts in patho-physiology and treatment. Cell Biosci. 4:27.

- Zhang, J., Chen, S., Hou, Z., Cai, J., Dong, M., & Shi, X., (2015). Perivascular-resident macrophage-like melanocytes in the inner ear are essential for the integrity of the intrastrial fluid-blood barrier. Proc Natl Acad Sci U S A.;109:10388–93. 10.1073/pnas.1205210109

- Zhang, J., Chen, S., Hou, Z., Cai, J., Dong, M., & Shi, X., (2015). Lipopolysaccharide-induced middle ear inflammation disrupts the cochlear intra-strial fluid-blood barrier through down-regulation of tight junction proteins. PLoS One, 10, e0122572.

- Zimmerlin L, Donnenberg VS, Rubin JP, Donnenberg AD, (2013). Mesenchymal markers on human adipose stem/progenitor cells. Cytometry. doi: 10.1002/cyto.a.22227. Epub 2012 Nov 26.

- Zozulya, A., Weidenfeller, C., & Galla, H. J., (2008). Pericyte-endothelial cell interaction increases MMP-9 secretion at the blood-brain barrier in vitro. Brain Research, 1189, 1–11.

List of Publications Dr Florinda Gennuso

- Isolation, cultivation, and characterization of primary bovine cochlear pericytes: A new in vitro model of stria vascularis

  Journal of Cellular Physiology

  2019 | journal-article

  DOI: 10.1002/jcp.27545EID: 2-s2.0-85054916994

- Immunohistochemical and molecular biomarkers in Coris julis exposed to environmental contaminants

  Ecotoxicology and Environmental Safety

  2010 | journal-article

  DOI: 10.1016/j.ecoenv.2009.12.025EID: 2-s2.0-77953610867

- Estrogen, neuroinflammation and neuroprotection in Parkinson's disease: Glia dictates resistance versus vulnerability to neurodegeneration

  Neuroscience

  2006 | journal-article

  DOI: 10.1016/j.neuroscience.2005.07.060EID: 2-s2.0-33644526277

- Glucocorticoid receptor-nitric oxide crosstalk and vulnerability to experimental parkinsonism: Pivotal role for glia-neuron interactions

  Brain Research Reviews

  2005 | journal-article

  DOI: 10.1016/j.brainresrev.2004.12.030EID: 2-s2.0-20244369883

- Hormones are key actors in gene X environment interactions programming the vulnerability to Parkinson's disease: Glia as a common final pathway

  Annals of the New York Academy of Sciences

  2005 | book

  DOI: 10.1196/annals.1356.023EID: 2-s2.0-32244434803

- Bilirubin protects astrocytes from its own toxicity by inducing up-regulation and translocation of multidrug resistance-associated protein 1 (Mrp1)

  Proceedings of the National Academy of Sciences of the United States of America

  2004 | journal-article

  DOI: 10.1073/pnas.0308452100EID: 2-s2.0-10744221309

- Glucocorticoid receptor deficiency increases vulnerability of the nigrostriatal dopaminergic system: critical role of glial nitric oxide.

  The FASEB journal : official publication of the Federation of American Societies for Experimental Biology

  2004 | journal-article

  EID: 2-s2.0-1342332143

- The reproductive system at the neuroendocrine-immune interface: Focus on LHRH, estrogens and growth factors in LHRH neuron-glial interactions

  Domestic Animal Endocrinology

  2003 | journal-article

  DOI: 10.1016/S0739-7240(03)00043-2EID: 2-s2.0-0141682415